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# THE ACTION OF HYPNOTIC DRUGS ON FROG SKELETAL MUSCLE

BY

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*From the Department of Pharmacology, King's College, London*

(RECEIVED MARCH 22, 1954)

Barbiturate drugs with a short duration of action are used extensively both clinically and in animal experiments, and it is of importance to know how they modify the responses of the skeletal neuromuscular junction. Preliminary experiments showed that barbiturates could augment the "twitch" of isolated frog muscles excited by their motor nerves, so it was decided to study their action upon frog muscle in greater detail.

The ilio-fibularis muscle was used, since Kuffler and his associates (see Kuffler, 1946, 1953) had studied in detail its dual innervation by both large and small diameter motor nerve fibres. The isolated muscle responds with a twitch to electrical stimulation of its motor nerve, and with a contracture to acetylcholine (ACh) added to the fluid bathing it. An appraisal of the pharmacological reactions of this preparation was therefore of considerable interest.

Evidence is presented that the sodium salts of thiopentone, pentobarbitone and hexobarbitone augment the twitch of the ilio-fibularis muscle in response to either indirect or direct stimulation and at the same time decrease or abolish the ACh contracture. These effects are compared with the actions of the barbiturates on the nerve-sartorius and rectus abdominis preparations of the frog, and on the phrenic nerve-diaphragm preparation of the rat. The action of some non-barbiturate hypnotics on muscle is also reported. Some of the results were communicated to the British Pharmacological Society in January, 1952, and January, 1953.

## METHODS

The ilio-fibularis muscle was exposed in the dorsum of the frog's thigh by retraction of the vastus externus and the semi-membranosus muscles. Its fascial sheath was carefully stripped, avoiding injury to its motor nerve—a fine branch of the sciatic entering the muscle in its proximal one-third on its antero-medial aspect. The muscle, with its nerve and parent sciatic trunk, was isolated and transferred to a 10 ml. bath of oxygenated frog Ringer solution at room temperature. Condenser discharges, through platinum electrodes, provided maximal stimuli at 15 sec. intervals to the sciatic nerve, or at 30 sec. intervals to the extremities of the muscle directly (M). The contractions to electrical stimuli, and the ACh contractures, were recorded isotonically by a weighted gimbal-lever. The muscle was loaded at 2 g., the amplification of the lever system being tenfold. ACh, acting for 60 sec., produced a contracture (Fig. 1). Responses to electrical stimulation, and sensitivity to ACh, were well maintained for many hours.

With the non-barbiturate hypnotics, single maximal stimuli were applied every 15 sec., alternately to the nerve and to the muscle, and the twitches, initially of equal height from both forms of stimulation, were recorded isotonically. A similar method was used for the nerve-sartorius preparation.

The technique used for the isolated frog rectus abdominis muscle was described by Quilliam and Strong (1949) and that for the isolated rat phrenic nerve-diaphragm preparation was a modification of Bülbring's (1946) method but with Krebs solution aerated with 5% $CO_2$ and 95% $O_2$.

## RESULTS

### *The Isolated Frog Sciatic Nerve Ilio-fibularis Muscle Preparation.*

In the normal muscle the twitches in response to both maximal indirect and direct stimulation were equal in height; 50 $\mu$g. of ACh produced a contracture.

*Thiopentone Sodium.*—Soon after adding the drug, in a concentration of $5 \times 10^{-5}$, there was a slow progressive increase in the contractions elicited by indirect stimulation, reaching a maximum in about 45 min. At this time the response to 50 $\mu$g. ACh was markedly reduced, though the addition of 1 mg. ACh produced a large contracture.

When the barbiturate concentration was increased to $1 \times 10^{-4}$, a further augmentation of the responses to nerve and to direct stimulation occurred; after 25 min. the addition of 50 $\mu$g. ACh produced a transient increase in the twitches but no contracture. The contracture caused by 1 mg. ACh was reduced

to that seen with the normal muscle after only 50 $\mu$g. ACh—a twentyfold decrease in ACh sensitivity. When the thiopentone concentration was raised to $2 \times 10^{-4}$, a further increase of the twitch occurred, but 1 mg. ACh now failed to produce contracture and temporarily reduced the muscle twitches. The contracture, originally evoked in the normal muscle by 10 mg. potassium chloride (KCl), was also abolished. Further increases of thiopentone concentration (to $3 \times 10^{-4}$, $5 \times 10^{-4}$ and to $1 \times 10^{-3}$) produced little change in the height of the muscle twitches; 1 mg. ACh no longer caused contracture, but temporarily decreased the twitch responses. At a final concentration of $2 \times 10^{-3}$, there was a sharp decline in the responses to nerve stimulation, terminating in complete neuromuscular block. At this stage the direct muscle response was reduced to about one-half of the maximal twitch height seen. Repeated washing now partially restored the muscle twitch.

*Pentobarbitone Sodium.*—The augmentation of the muscle twitch with pentobarbitone sodium (Fig. 1) was considerably less than with thiopentone. At a concentration of $6 \times 10^{-4}$, neuromuscular block appeared—becoming complete at a concentration of $1.4 \times 10^{-3}$. With pentobarbitone, partial neuromuscular block occurred at a lower concentration ($6 \times 10^{-4}$) than with thiopentone. No reduction was seen in the direct muscle response until the pentobarbitone concentration reached $1.4 \times 10^{-3}$. At this stage repeated washing restored the muscle responses to direct and indirect stimulation. The contracture from 20 $\mu$g. ACh was decreased in stages as the concentration of pentobarbitone was increased, and was abolished only at $6 \times 10^{-4}$. At the highest concentration attained ($1.4 \times 10^{-3}$), 1 mg. ACh still evoked a contracture, but this was considerably smaller than that with 10 $\mu$g. ACh in the fresh muscle. With this barbiturate concentration, no contracture occurred with 5 mg. of KCl.

*Hexobarbitone Sodium.*—With this drug, the augmentation of the twitch response to indirect and direct stimulation followed the pattern seen with pentobarbitone sodium except that the neuromuscular block, which appeared at $1 \times 10^{-3}$, was never complete—even at the highest concentration used ($1.5 \times 10^{-3}$), when the indirect response was reduced to about 60% of the direct response. The contracture elicited by 25 $\mu$g. ACh was less readily suppressed than with pentobarbitone, and only at the highest hexobarbitone concentration was it abolished; at this stage 1 mg. ACh produced a contracture much smaller than did a test dose of 25 $\mu$g. in the fresh muscle.

*Comparison of Effects of Barbiturates on the Muscle Twitch and on the ACh Contracture.*—In Fig. 2 the increase in the muscle twitch in response to indirect stimulation is plotted against the con-

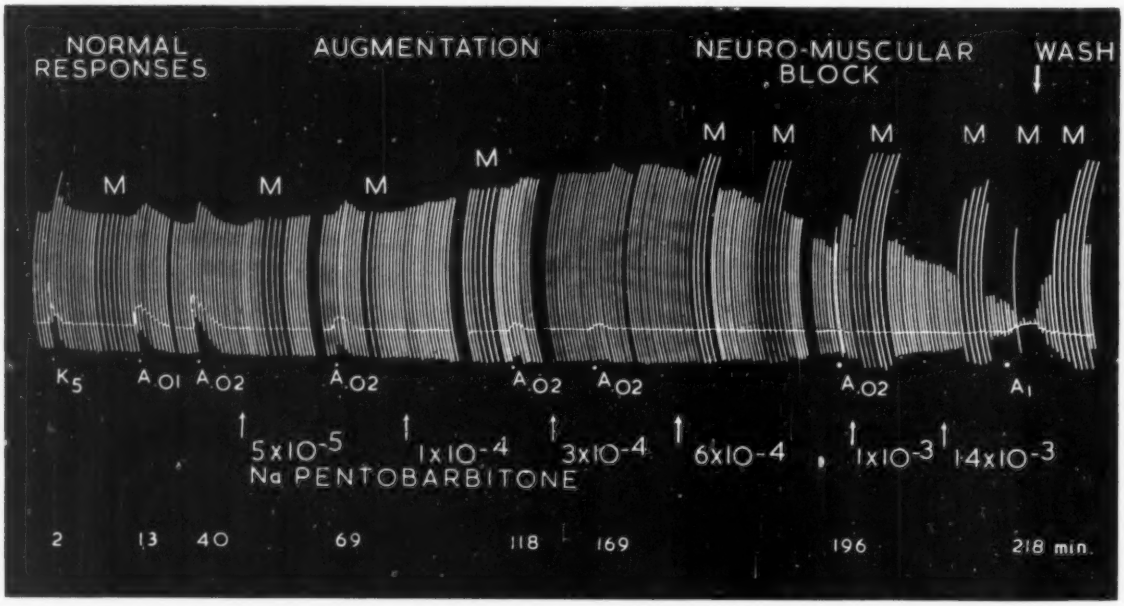


FIG. 1.—Isolated frog sciatic nerve ilio-fibularis muscle preparation. Record of isotonic twitches evoked by indirect or direct (M) single maximal stimuli. The contracture response to 0.01 mg. (A 0.01), 0.02 mg. (A 0.02), and 1 mg. (A1) ACh and to 5 mg. (K5) KCl are recorded at intervals. The record illustrates the action of pentobarbitone sodium which first augments the muscle twitch; it then produces neuromuscular block and decreases the contracture response to added ACh.

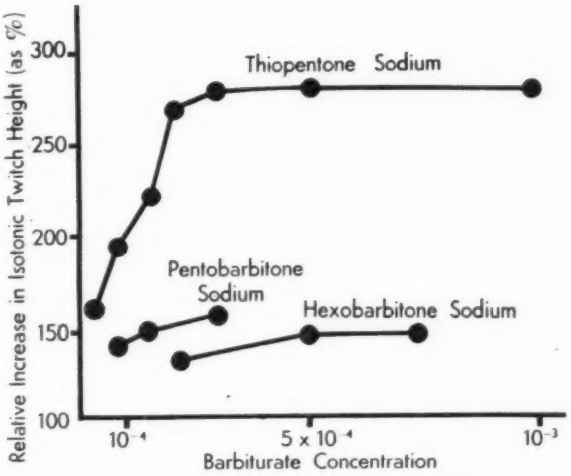


FIG. 2.—Isotonic twitches were recorded from the isolated frog ilio-fibularis muscle stimulated indirectly. The graph shows the relation between concentration of barbiturate and percentage increase in twitch height.

centration of barbiturate. The graphs were constructed from measurements obtained from Fig. 1 and from typical experiments with thiopentone and hexobarbitone.

The twitch heights progressively increased as the drug concentrations rose. With each barbiturate, a concentration was reached above which no further augmentation occurred. Thiopentone sodium differed from the other two compounds in giving nearly twice as much augmentation.

The contracture elicited by a small dose of ACh (usually 20 to 50 μg./10 ml.) was decreased by low and abolished by high concentrations of the three barbiturates. The effects of 1 mg. ACh were decreased by high concentrations of all three barbiturates, but were abolished only by thiopentone sodium. Thiopentone was thus the most active agent in reducing the ACh contracture, pentobarbitone was intermediate, and hexobarbitone was the least active.

*Other Isolated Muscle Preparations*

Experiments with the frog sartorius and rectus and the rat diaphragm muscles were conducted along the same general lines as those used with the ilio-fibularis muscle. In most instances, 4 to 7 experiments were made to provide each average figure shown in Table I.

The order of activity of the barbiturates in producing firstly an initial and secondly a maximum increase in twitch height in all muscles examined was thiopentone, then pentobarbitone and lastly hexobarbitone. In the production of neuromuscular block, the most active drug was, with the ilio-fibularis and the diaphragm, pentobarbitone and, with the sartorius, thiopentone. No neuromuscular block could be obtained with thiopentone in the rat diaphragm, since the responses to both direct and indirect stimulation were decreased equally as the concentration rose, the strength chosen for direct muscle stimulation being sufficient to excite a fully curarized muscle.

*Other Barbiturates*

Other barbiturates were less active than the three just described in producing the characteristic augmentation of the muscle twitch response and a reduction of the acetylcholine contracture in frog muscle (Table II). Even with the rectus, which was the most sensitive of the three test tissues, the concentrations of barbiturates required were too high to permit detailed quantitative study. Large doses of the barbiturates listed in Table II produced an augmentation of the twitch response in the rat diaphragm stimulated via its nerve.

*Control Experiments with the Isolated Frog Sartorius and the Ilio-fibularis Muscle Preparations*

As dilute solutions of the sodium salts of barbiturates are alkaline, measurements were made of the effect of sodium and hydroxyl ions on the

TABLE I

EFFECTS OF BARBITURATES ON MUSCLE TWITCH RESPONSES

| Average Dose Required to Cause: | Thiopentone Sodium | | | Pentobarbitone Sodium | | | Hexobarbitone Sodium | | |
|---|---|---|---|---|---|---|---|---|---|
| | Initial Increase in Twitch Height (μg./ml.) | Maximum Increase in Twitch Height (μg./ml.) | Neuro-muscular Block (μg./ml.) | Initial Increase in Twitch Height (μg./ml.) | Maximum Increase in Twitch Height (μg./ml.) | Neuro-muscular Block (μg./ml.) | Initial Increase in Twitch Height (μg./ml.) | Maximum Increase in Twitch Height (μg./ml.) | Neuro-muscular Block (μg./ml.) |
| Frog muscle: | | | | | | | | | |
| Ilio-fibularis .. | 50 | 150 (250) | 2,000 | 50 | 300 (150) | 570 | 300 | 1,000 (140) | 680 |
| Rectus abdominis .. | 25 | 310 (330) | | 100 | 1,500 (230) | | 200 | 2,500 (200) | |
| Sartorius .. .. | 25 | 50 (160) | 100 | 100 | 500 (150) | 500 | 100 | 1,000 (150) | 1,000 |
| Rat muscle: | | | | | | | | | |
| Diaphragm .. | 12·5 | 85 (150) | | 17·5 | 172 (120) | 220 | 98 | 440 (140) | 570 |

The figures in parentheses indicate the maximum % increase in twitch height.

TABLE II

AVERAGE THRESHOLD CONCENTRATIONS REQUIRED TO PRODUCE AUGMENTATION OF THE MUSCLE TWITCH

| | Frog Muscle | | Rat Muscle |
|---|---|---|---|
| | Ilio-fibularis (mg./ml.) | Rectus (mg./ml.) | Diaphragm (mg./ml.) |
| Butobarbitone sodium .. | 20* | 2* | 0·16 |
| Allobarbitone „ .. | 30* | 4* | 0·16 |
| Barbitone „ .. | 50* | 10 | 0·60 |
| Phenobarbitone „ .. | 50 | 10 | 0·30 |

* Indicates that ACh-contracture was decreased at this concentration.

muscle twitch. When the sodium chloride concentration was raised by 1 mg./ml. in the frog Ringer solution bathing a fresh muscle, there was little change in the response to either direct or indirect stimulation. When the sodium bicarbonate content of the Ringer solution was raised by 100 μg./ml. no alteration of the responses of the muscle occurred, although larger amounts (1 mg./ml.) decreased the twitch elicited by nerve or muscle stimulation, an effect which was completely reversed by washing. If the *p*H of the Ringer solution was raised by adding 100 μg./ml. sodium hydroxide, there was an abrupt augmentation of the responses to both types of stimulation. When the *p*H was returned to 7.2 by bubbling a mixture of 5% $CO_2$ and 95% $O_2$ through the bath liquid, the height of the muscle twitches returned to the original level. However, larger quantities of sodium hydroxide (200 μg./ml.) decreased both types of muscle response, but adjustment of the *p*H to 7.2 restored the original amplitude of the twitches.

If pentobarbitone sodium (final concentration 100 μg./ml.) was added to the Ringer bathing a fresh muscle, the *p*H of the fluid rose above 8.8 and the muscle twitches in response to nerve and muscle stimulation were augmented equally. When this augmentation had reached its maximum, adjustment of the *p*H to 7.2 with $CO_2$ produced a further augmentation of the responses to both types of stimulation. With higher concentrations of pentobarbitone, it was not possible to return the bath fluid to *p*H 7.2 with $CO_2$. Similar effects were observed with thiopentone and hexobarbitone.

*Non-Barbiturate Hypnotic Drugs*

*Paraldehyde*, added to the fluid bathing a fresh ilio-fibularis muscle, to give a final concentration of 0.5 mg./ml., caused a slight decrease in the height of the muscle twitch elicited by a single maximal stimulus applied to the motor nerve. As the paraldehyde concentration was raised, neuromuscular block increased and became complete at 1.0 to 1.5 mg./ml.; the response to direct muscle stimulation remained unchanged. When the amplitude of the indirect response had fallen to a constant value, the addition of 50 μg. of ACh, or of 50 μg. of decamethonium iodide, caused a transient increase in twitch height. The block from paraldehyde was not relieved by eserine, but washing restored the normal response of the preparation.

*Methylpentynol* exerted an action upon the ilio-fibularis muscle similar to that seen with paraldehyde. The addition of 0.5 mg./ml. caused a small degree of neuromuscular block (Fig. 3). When the concentration was increased to 1.0 mg./ml., a more rapidly developing neuromuscular block set in which could be partly relieved, but only for the short period of 5 min., by adding either 50 μg. decamethonium or 50 μg. ACh to the 10 ml. bath. As with paraldehyde, prolonged exposure to decamethonium always produced an increase of the neuromuscular block. After washing in frog Ringer solution, the amplitude of the muscle twitch in response to nerve stimulation was restored to that seen in the fresh muscle.

*Chlorbutol* was similar in action to paraldehyde except that it produced a complete neuromuscular block in a concentration of only 0.5 mg./ml.

*Urethane* produced neuromuscular block at 5 mg./ml., and was thus similar to, but less potent than, paraldehyde.

*Chloralose* in concentrations of 1.5 to 2.5 mg./ml. caused a progressive augmentation of the muscle twitch in response to both direct and indirect

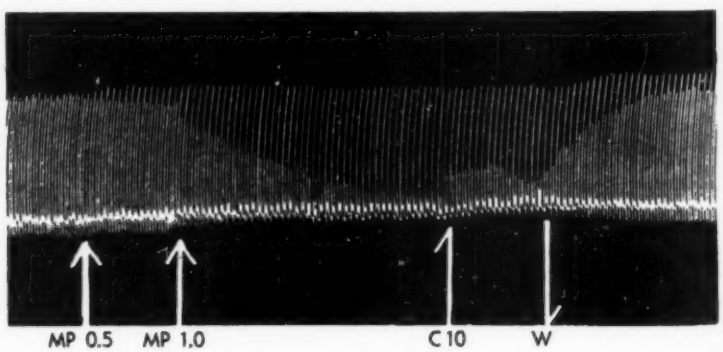


FIG. 3.—The isolated frog sciatic nerve ilio-fibularis muscle preparation. A single maximal stimulus was applied every 15 sec. alternately to the nerve or to the muscle directly. Methylpentynol 0.5 mg./ml. (MP 0.5) and 1 mg./ml. (MP 1.0) caused neuromuscular block which was partly relieved by 50 μg. of decamethonium (C10). Washing (W) restored the responses. Bath volume—10 ml.

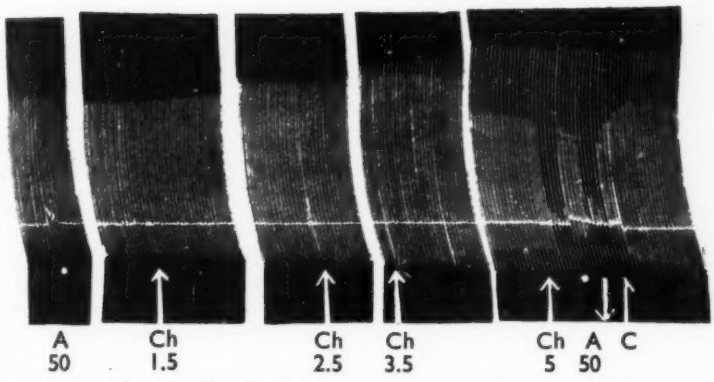


FIG. 4.—Record as in Fig. 3. Chloralose 1.5 mg./ml. (Ch 1.5) and 2.5 mg./ml. (Ch 2.5) caused an augmentation of the muscle twitch in response to indirect or direct stimulation. With 3.5 mg./ml. (Ch 3.5) and 5.0 mg./ml. (Ch 5), a neuromuscular block developed which was partly but transiently relieved by 50 μg. ACh (A50) or by 25 μg. decamethonium (C). At downward pointing arrow, the muscle was washed with frog Ringer solution containing 5 mg./ml. of chloralose. The contracture following ACh (A50) was only slightly reduced by chloralose.

stimulation, like the barbiturates. With higher concentrations there was a further augmentation of the response to direct stimulation but a decline of that to nerve stimulation. At a concentration of 5 mg./ml. complete neuromuscular block developed rapidly; it was reversed immediately but temporarily by 50 μg. ACh or by 25 μg. decamethonium. Fig. 4 illustrates these effects. Washing removed the effects of chloralose.

The effects of *carbromal*, in concentrations of 0.06 mg./ml. and 0.12 mg./ml., are shown in Fig. 5. The responses to both forms of stimulation were at first augmented; neuromuscular block then appeared and soon became complete. The responses to direct stimulation continued to be augmented, reaching nearly twice their initial value, an effect that was particularly marked with carbromal. At this stage, neither 50 μg. ACh, 50 μg. decamethonium, or 100 μg. eserine, relieved the block though they did so at lower concentrations of the hypnotic. Washing usually restored the responses of the preparation to their original size.

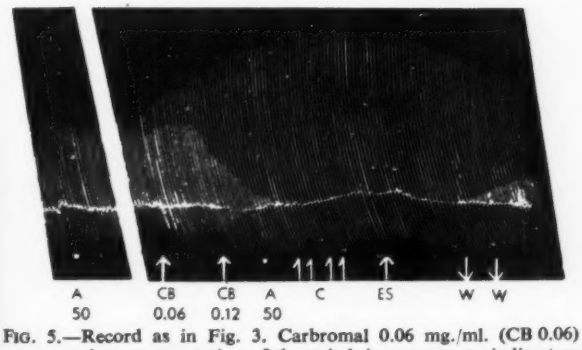


FIG. 5.—Record as in Fig. 3. Carbromal 0.06 mg./ml. (CB 0.06) caused an augmentation of the twitch in response to indirect or direct stimulation. Carbromal 0.12 mg./ml. (CB 0.12) produced a neuromuscular block associated with a marked augmentation of the direct muscle response. Neither 50 μg. ACh (A50), four doses of 50, 50, 100, and 100 μg. decamethonium (C) nor 100 μg. eserine (ES) relieved the block. ACh and decamethonium were followed by a contracture. Washing (W) restored the responses.

*Chloral hydrate* did not cause neuromuscular block but, in doses of 0.2 to 2.5 mg./ml., decreased equally the twitches evoked by direct and by indirect stimulation.

Non-barbiturate drugs, then, in concentrations which produced a marked degree of neuromuscular block, caused little change in the ACh contracture of the ilio-fibularis muscle.

In the rat diaphragm, paraldehyde, methylpentynol, chloralose and carbromal produced neuromuscular block in concentrations comparable with those required to block the amphibian neuromuscular junction.

## DISCUSSION

One action of barbiturates upon isolated frog skeletal muscles was to augment the twitch arising from nerve stimulation. A similar effect was observed with the rat diaphragm. Other workers have also seen this response in mammalian muscle (Huston, Martin, and Dille, 1947; Riedel and Huston, 1949; Secher, 1951; Kraatz, Gluckman, and Shields, 1953). Thus Secher (1951) reported this action with the rat diaphragm and Huston, Martin, and Dille (1947) observed an increase in the contractions of the gastrocnemius muscle elicited by indirect stimulation when pentobarbitone was added to the defibrinated blood perfusing a dog's isolated hind limb. In the rat, intraperitoneal administration of amylobarbitone sodium was followed by an increased twitch from the sciatic nerve-gastrocnemius muscle preparation (Riedel and Huston, 1949) and Kraatz, Gluckman, and Shields (1953) saw, with thiopentone and pentobarbitone, a similar augmentation in tension in the indirectly stimulated anterior tibial muscles of cats and dogs.

The depression of the ACh contracture in frog muscle with barbiturates has received no attention except for the report of Torda and Wolff (1947), who demonstrated a reduction in the ACh contracture of the rectus abdominis muscle with pentobarbitone and amylobarbitone.

The control experiments showed that neither sodium nor hydroxyl ions contributed to the aug-

mentation by barbiturates of the twitch heights of isolated frog muscles, but that these ions would be expected to oppose the observed muscle responses. The simple gimbal lever, lightly weighted, was used to record the isotonic twitches in isolated frog muscles because concurrent records of the ACh contractures were required. The mechanical limitations of such a lever system must not be overlooked even though they were reduced by the strict application of a comparative technique.

TABLE III

A CLASSIFICATION OF HYPNOTIC DRUGS BASED UPON THEIR ACTION UPON THE MUSCLE TWITCH RESPONSE OF THE ISOLATED SCIATIC NERVE ILIO-FIBULARIS MUSCLE PREPARATION

| Group | Hypnotic | Threshold Concentration (mg./ml.) |
|---|---|---|
| (*a*) Augmentation of muscle twitches evoked by indirect and direct stimulation | Thiopentone sodium<br>Pentobarbitone ,,<br>Hexobarbitone ,, | 0·05<br>0·05<br>0·30 |
| (*b*) Neuromuscular block with response of muscle to direct stimulation unaltered | Chlorbutol<br>Paraldehyde<br>Methylpentynol<br>Urethane | 0·1<br>0·5<br>0·5<br>5·0 |
| (*c*) Neuromuscular block with an augmented response from direct muscle stimulation | Carbromal<br>Chloralose | 0·12<br>3·50 |
| (*d*) Depression of twitches in response to indirect and direct stimulation | Chloral hydrate | 0·20 |

Whereas the first action of barbiturates upon amphibian and mammalian muscle was to produce an augmentation of the muscle twitch, that of non-barbiturate hypnotics was to impair neuromuscular transmission. On the basis of these peripheral actions, it was possible to divide hypnotics into four main classes (Table III). Both urethane in group (*b*) and chloralose in group (*c*) are distinguished by their low neuromuscular blocking activity.

The actions of hypnotics at the skeletal neuromuscular junction may be regarded as side effects. It was of some interest to consider what relation the concentrations used in these experiments bore to the mean human pharmacopoeial dose, expressed either as mg./kg. in a man weighing 70 kg. or as a concentration in mg./ml., assuming that it might be theoretically possible to distribute the dose equally throughout a blood volume of 5 l. In Table IV an estimate of these concentrations is made. The ratio of the human therapeutic dose (mg./kg.) to the threshold dose of the hypnotic (mg./ml.) required to produce an effect in the isolated frog ilio-fibularis muscle ranges, with four exceptions, between 77.5 and 143: with hexobarbitone it is 29, with methylpentynol 14.3, with urethane 4.3, and with chloralose 1.6. There is a positive correlation between the theoretical blood concentration and the *in vitro* dose required, except with the four drugs just mentioned. The discrepancy with methylpentynol might arise from too low an estimate of the human therapeutic dose: further experience with this hypnotic in man might decide this point. The larger discrepancies with urethane and chloralose are specially interesting, for these two compounds are used to produce long-lasting general anaesthesia in animals. Exley (1954) found that chloralose was almost devoid of ganglion-blocking activity. Thus chloralose commends itself as a general anaesthetic in laboratory experiments in which neuromuscular or ganglionic transmission is to be studied, the drug being virtually without action on both structures.

*Mechanism of Action of Hypnotics on Muscle*

The augmentation by barbiturates of the muscle twitch elicited by indirect stimulation might be

TABLE IV

RELATION OF HUMAN THERAPEUTIC DOSE TO THRESHOLD CONCENTRATION REQUIRED FOR AN ACTION ON THE ISOLATED FROG ILIO-FIBULARIS MUSCLE PREPARATION

The mean of the human therapeutic dose is expressed both as mg./kg. in a man weighing 70 kg. and as the concentration of the hypnotic which might be attained if it were theoretically possible to distribute it equally throughout a blood volume of 5 l.

| Group and Compound | Mean of Human Therapeutic Dose | | Theoretical Concentration in Blood mg./ml. | Threshold Concentration for Action on Ilio-fibularis mg./ml. | Ratio Human Therapeutic Dose (mg./kg.)/ Threshold Dose for Ilio-fibularis |
|---|---|---|---|---|---|
| | g. or ml. | mg./kg. | | | |
| (*a*) Thiopentone sodium | 0·3 | 4·3 | 0·06 | 0·05 | 86 |
| Pentobarbitone ,, | 0·3 | 4·3 | 0·06 | 0·05 | 86 |
| Hexobarbitone ,, | 0·6 | 8·6 | 0·12 | 0·3 | 29 |
| (*b*) Chlorbutol | 0·75 | 10·7 | 0·15 | 0·1 | 107 |
| Paraldehyde | 5·0 | 71·4 | 1·0 | 0·5 | 143 |
| Methylpentynol | 0·5 | 7·14 | 0·1 | 0·5 | 14·3 |
| Urethane | 1·5 | 21·4 | 0·3 | 5·0 | 4·3 |
| (*c*) Carbromal | 0·65 | 9·3 | 0·13 | 0·12 | 77·5 |
| Chloralose | 0·4 | 5·7 | 0·08 | 3·50 | 1·6 |
| (*d*) Chloral hydrate | 1·6 | 23·1 | 0·32 | 0·20 | 115 |

expected to arise from either an anticholinesterase activity or an altered response of the muscle cell. The observed depression by barbiturates of the ACh contracture affords evidence that an anticholinesterase action is not implicated. As the effect of thiopentone is consistent with a decrease in the rate of propagation of the muscle action potential in the rat gracilis muscle (Quilliam, 1955), the mode of action of barbiturates on the " twitch muscle system " appears to reside in altered response of the muscle fibre.

The neuromuscular block caused by non-barbiturate hypnotics could arise from a decreased production of the local hormone at the nerve endings, an increased cholinesterase activity, a competitive blocking action or a long-lasting depolarization of the muscle fibre in the region of the neuromuscular junction. The present experiments throw no light upon the ACh release at the nerve endings nor upon the depolarizing activity of the drugs ; but the absence of a material change in the amplitude of the ACh contracture appears to eliminate increased cholinesterase activity as a major cause. The relief of the block by added ACh or decamethonium lends support to the view that the block is competitive in nature, but the absence of an effect with eserine in this connexion is puzzling. The mechanism of the augmentation of the response to direct stimulation probably resides in an altered response on the part of the muscle fibre itself because of the neuromuscular blocking action of the non-barbiturate hypnotics.

Before a final view of the mode of action of these groups of hypnotics upon the skeletal neuromuscular junction can be taken, a further study of the electrical changes associated with the action of these drugs upon the muscle cell must be undertaken.

The present work shows that the isolated frog sciatic nerve ilio-fibularis muscle preparation can be used to provide concurrent information on the contracture response evoked from frog muscle by ACh added to the bath fluid and on the twitch elicited by maximal stimulation of the motor nerve or of the muscle directly. Although it is outside the province of this paper to discuss in detail the relationship between these responses and those of the dual skeletal nerve-muscle systems in the frog (Kuffler, 1953), it is sufficient here to draw an analogy between the known effect of stimulation of the " slow skeletal muscle system " and the addition of ACh on the one hand, and between the effect of stimulation of the " twitch system " and the response of the ilio-fibularis muscle to single stimuli applied to its motor nerve on the other hand, and to point out the action of drugs on these effects.


## Summary

1. The actions of the sodium salts of thiopentone, pentobarbitone, and hexobarbitone, upon the contractures elicited by ACh, and upon twitches evoked by electrical stimulation in the isolated frog sciatic nerve ilio-fibularis muscle, were compared with those in the isolated frog motor-nerve sartorius and the rectus abdominis muscle preparations.

2. In the frog ilio-fibularis, the contractures elicited by small doses of ACh were reduced by low and abolished by high concentrations of the three barbiturates. The contracture from a large dose of ACh was decreased by high concentrations of the three barbiturates but was abolished only by thiopentone. In these actions thiopentone was the most active agent ; pentobarbitone was next, and hexobarbitone was least.

3. The three barbiturates augmented the height of the muscle twitch elicited by indirect electrical stimulation of isolated frog muscles and of the isolated rat diaphragm. In this direction also, thiopentone was the most active, hexobarbitone the least active, with pentobarbitone occupying an intermediate position. High concentrations of the three barbiturates caused a block in neuromuscular transmission in all the muscles except the rat diaphragm, in which thiopentone reduced the responses to indirect and direct stimulation equally.

4. Large doses of the sodium salts of butobarbitone, allobarbitone, barbitone and phenobarbitone reduced the ACh contracture in frog muscle and augmented the twitch in response to indirect and direct electrical stimulation in frog and rat muscles.

5. Non-barbiturate hypnotics, such as paraldehyde, methylpentynol, chlorbutol and urethane, produced a neuromuscular block in concentrations which cause little, if any, reduction of the ACh contracture in the frog ilio-fibularis muscle. With chloralose and carbromal, the neuromuscular block is associated with an augmentation of the muscle twitch following direct electrical stimulation.

6. A classification of hypnotics is proposed, based upon their ability to augment or to reduce the height of the muscle twitch in response to nerve stimulation. The significance of these effects and the possible modes of action of these drugs are discussed.

7. The high concentrations of chloralose and of urethane required to reduce the response to nerve stimulation commend them as general anaesthetics for laboratory animals when studies of neuromuscular transmission are being made.

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# THE ACTION OF THIOPENTONE SODIUM ON SKELETAL MUSCLE

BY

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(RECEIVED APRIL 26, 1954)

Of those barbiturates which have a short duration of action as central nervous system depressants, thiopentone sodium was the most active in augmenting the muscle twitch elicited by single maximal electrical stimuli applied to the motor nerves of frog or rat nerve-muscle preparations (Quilliam, 1955). Analysis of the pharmacological data obtained from these isolated tissue studies left unsolved the precise mechanism of this barbiturate effect. It seemed that an examination of the changes in the muscle action potential of the rat anterior gracilis muscle, excited through its motor nerve before and after the intra-arterial injection of thiopentone sodium, might shed some light upon the mode of action of barbiturates.

The experiments reported in this paper show that, after the intra-arterial injection of thiopentone sodium, the voltage of the muscle action potential was reduced, its latency was increased and its duration was prolonged—these changes being consistent with a decreased rate of propagation of the potential over the surface of the muscle fibre. The significance of these changes and their relation to the augmentation of the muscle twitch are discussed. Some of the results were communicated to the British Pharmacological Society in July, 1952.

## METHODS

*Rat Sciatic Nerve Gastrocnemius-Soleus Preparation.*—In adult rats, lightly anaesthetized with 1.2 g./kg. of urethane intraperitoneally, the tendo Achillis was carefully freed from the surrounding tissues and attached to an isometric spring steel myograph. The sciatic nerve was exposed in the thigh and severed. Its cut peripheral stump was stimulated maximally by suitable condenser discharges applied through platinum electrodes at the rate of one/sec. and the muscle twitches recorded upon a smoked drum.

*Rat Obturator Nerve Anterior Gracilis Preparation.*—The anterior gracilis muscle was exposed by a minimum of dissection in the thigh of a rat lightly anaesthetized with urethane administered intraperitoneally. The obturator nerve supplying it was prepared for stimulation with platinum electrodes (Jarcho, Eyzaguirre, Berman, and Lilienthal, 1952). Skin flaps were raised so that a pool of liquid paraffin maintained at 39° C. covered the area exposed. Under these conditions, single maximal stimuli (condenser discharges) applied to the nerve at the rate of one/sec. evoked muscle action potentials, all of which appeared to have the same characteristics for long periods of time. A branch of the abdominal aorta, or the contralateral femoral artery, was prepared so that thiopentone sodium could be injected into the blood supplying the muscle with the minimum of disturbance to the preparation. Throughout each experiment single maximal stimuli were applied to the obturator nerve at the rate of one/sec.

Platinum electrodes, placed upon the superficial surface of the muscle, led off the muscle action potentials to the recording equipment. The amplifiers used were A.C. coupled and had a frequency response to a sine wave-form which was practically flat from 2 cycles/sec. up to 10 kcycles/sec. Thus the amplification system did not introduce appreciable frequency distortion of the muscle action potentials. The amplified potentials were displayed on and photographed from oscilloscopes.

Intra-arterial injections of 0.9% sodium chloride solution at body temperature altered neither the height of the muscle twitch nor the form of the muscle action potentials.

## RESULTS

*Rat Sciatic Nerve Gastrocnemius-Soleus Preparation.*—The intra-arterial injection of thiopentone caused a marked augmentation of the muscle twitches in response to single maximal stimuli applied to the sciatic nerve stump. In the experiment illustrated in Fig. 1, the injection of 2 mg. thiopentone sodium was followed by a rapid augmentation of the muscle twitch which became maximum within about 1 min. and, thereafter, the twitch height declined. In this experiment the respiration failed after about 7.5 min. However, if not more than 1 mg. was injected intra-arterially, the rats survived (see Figs. 3 and 5), but, with 1 to 2 mg. of the drug intravenously, respiratory failure usually followed within one minute.

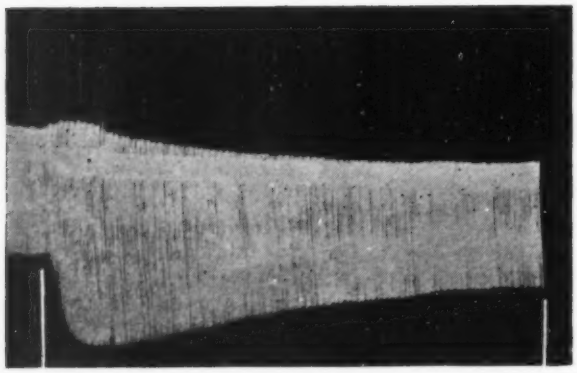

FIG. 1.—Rat sciatic nerve gastrocnemius-soleus muscle. Isometric records of the muscle twitch in response to single maximal stimuli applied to the sciatic nerve at 1/sec.; 2 mg. thiopentone sodium was injected arterially at first mark. Respiration failed at second mark, 7.5 min. later.

As the anatomical arrangement of the fibres in the soleus and gastrocnemius muscles was complex, the anterior gracilis muscle was selected for electrical studies.

*Rat Obturator Nerve Anterior Gracilis Preparation.*—The amplitude of the muscle twitch elicited by single maximal stimuli applied to the obturator nerve was augmented by intra-arterial thiopentone in a manner similar to that observed with the soleus-gastrocnemius muscle group.

The anterior gracilis muscle is about 35 mm. long, 5 mm. wide, and about 1 mm. thick, running as a parallel-sided strap across the thigh and with a thin flat tendon at each end (Fig. 2). Some muscle fibres appear to run the whole length of the muscle, while others do not, but all the fibres are arranged parallel one to another. The end-plates are gathered in two bands each about 1 mm. wide in the region of the junction of each one-third of the muscle. These are indicated by the letters E–E in Fig. 2. Thus, the muscle action potential recorded in any region may contain certain components arising from both end-plate concentrations, and may vary considerably from point to point along the muscle. When the amplifier leads were upon region C of the muscle (Fig. 2), the record contained, as a major diphasic component, the potential arising from the adjacent end-plate focus, followed by a minor component from the distant end-plate focus. Such a record is illustrated in Fig. 3 at time 0 min. in which a small late deflection was all that was seen of the muscle action potential arising from the distant end-plate focus. Similarly, the muscle action potentials recorded from region A were dominated by a large diphasic component from the local end-plate focus with a minor contribution from the distant focus.

In region B (Fig. 2), the contributions from each end-plate focus were nearly equal in value and the form of the muscle action potential recorded was quadriphasic as in Fig. 5. These results were similar to those of Jarcho *et al.* (1952).

In none of the electrical records made after intra-arterial thiopentone was there any evidence of repetitive muscle action potentials in response to maximal stimuli applied to the obturator nerve. Thus it seemed probable that some mechanism of action of the drug should be sought other than one involving an anticholinesterase action.

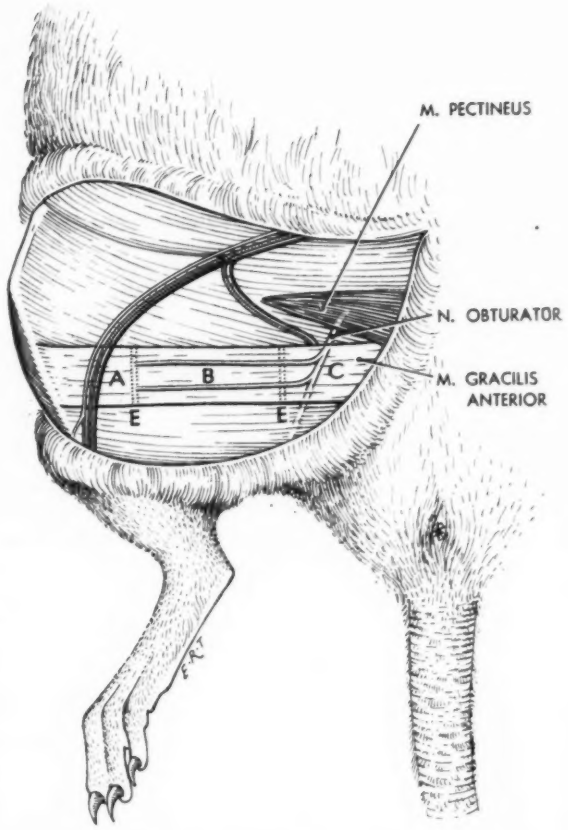


FIG. 2.—Diagram of the rat obturator nerve anterior gracilis muscle preparation. The end-plate regions are localized in two bands about 1 mm. wide denoted by E–E.

In Fig. 3, the muscle action potential was recorded at intervals from the same area in region C of Fig. 2 during a control period of 23 min. of maximal single stimuli applied to the obturator nerve at a frequency of 1/sec. In all, nearly 1,400 stimuli were given, but the form and voltage (amplitude) of the various components and the latencies (i.e., the intervals between the stimulus artifact and the various peaks) of the muscle action potential records remained constant throughout.

FIG. 3.—Action potentials from the rat anterior gracilis muscle in region C (Fig. 2); the obturator nerve was stimulated with maximal shocks at 1/sec. At 25 min. 1 mg. thiopentone sodium was injected intra-arterially and at 39 min. 3 mg. was injected. Oscillograms touched up.

After 1 mg. thiopentone sodium intra-arterially, examination of the peaks of the potentials recorded one and seven minutes later showed a slight decrease in voltage and an increase in latency. A larger dose (3 mg.), injected at 39 min., caused a considerable reduction in voltage and further increase in latencies of the peaks of the muscle action potential; this large dose caused a failure of respiration followed by cardiac arrest.

The graphs in Fig. 4 have been constructed from measurement of the potentials given in Fig. 3. The solid circles joined by solid lines indicate the increase in latency of peaks *a* and *b*, while the open circles and broken lines demonstrate the fall in voltage of peaks *a* and *b* after the two injections of thiopentone. It can be seen that neither the voltage nor the latency returned to the original values during

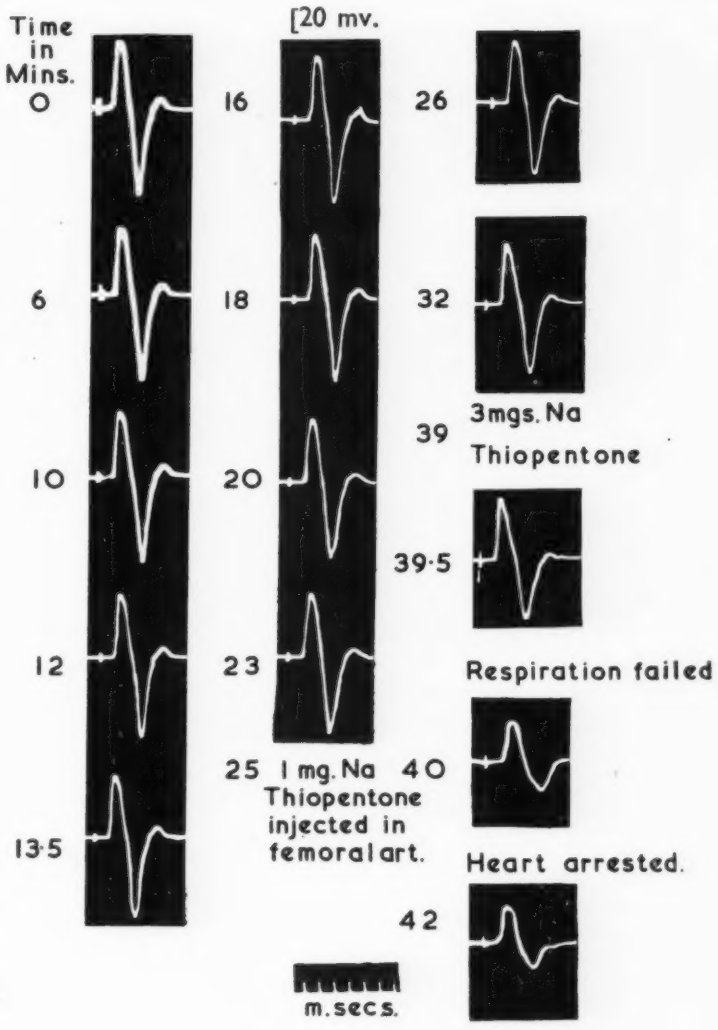


FIG. 3

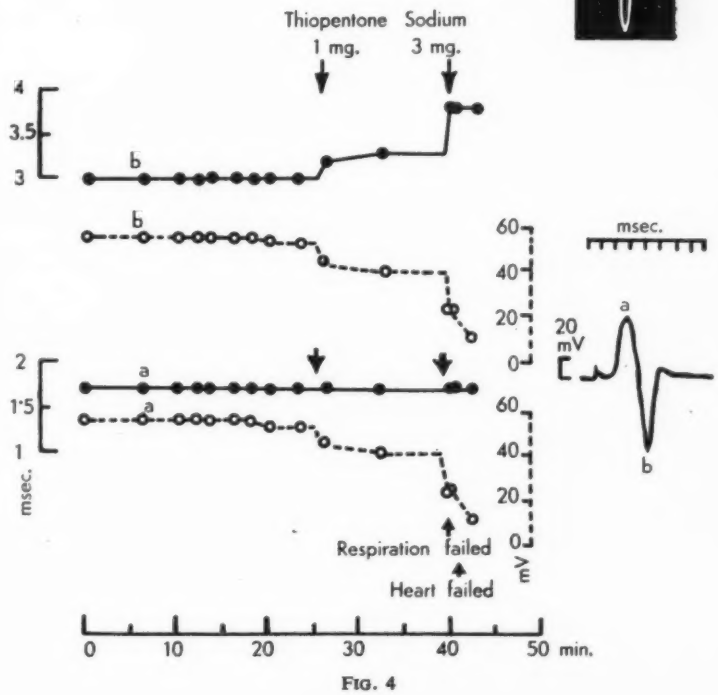


FIG. 4

the short interval between the two injections. At least one hour elapsed before the voltage and the latency of the potentials returned near to normal (Fig. 6).

In another experiment, the muscle action potential, recorded from a point upon the surface of region B (Fig. 2) of the anterior gracilis muscle, presented four peaks for analysis. The injection of 1 mg. thiopentone sodium into the abdominal aorta at 3 min.

FIG. 4.—Summary of experiment depicted in Fig. 3. The open circles represent the voltages of peaks *a* and *b* of the muscle action potential and the solid circles represent the latencies of peaks *a* and *b*.

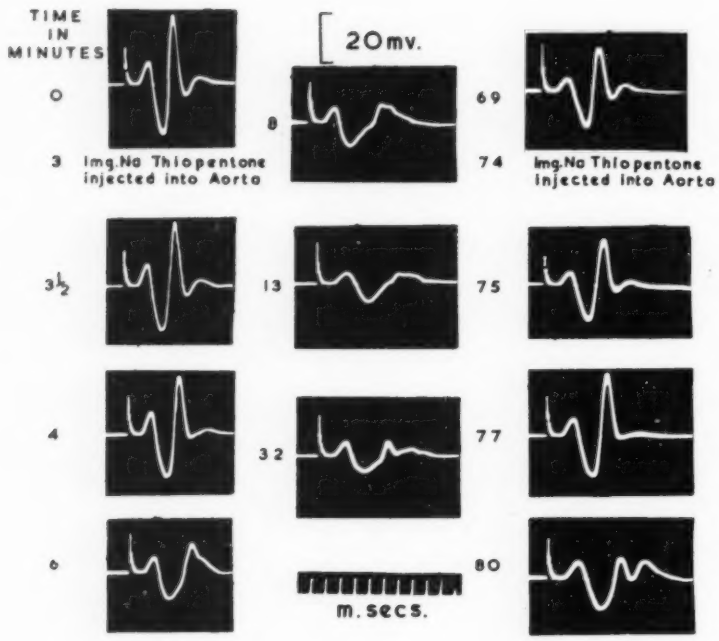


FIG. 5.—Rat obturator nerve anterior gracilis muscle preparation. The muscle action potentials were recorded from a point in region B (Fig. 2). The obturator nerve was stimulated 1/sec.; at 3 min. and at 74 min. 1 mg. thiopentone sodium was injected into the abdominal aorta. Oscillograms touched up.

produced an increase in latency and a decrease of the voltage of all four peaks (Fig. 5); these changes were maximal at about 10 min. after the injection. Recovery of the potentials began after 30 min., and even 66 min. after the injection was not quite complete.

A second injection of 1 mg. thiopentone sodium produced a similar sequence of events. At 77 min. the voltage of the third peak was greater than it was immediately after this injection—a change not seen after the first injection; the last peak of the complex, moreover, was diminished.

The increase in latency and the fall in voltage of each peak of the action potential, as well as its total duration, are expressed graphically in Fig. 6.

## DISCUSSION

The results reported in this paper show that intra-arterial injection of thiopentone sodium into rat skeletal muscle augments the muscle twitch elicited by maximal stimuli applied to the motor nerve. The mechanism underlying this effect might be thought to be an anticholinesterase action of the barbiturate promoting the accumulation of the transmitter substance. Such an accumulation might be expected to result in repetitive firing of the muscle action potential after a single nerve impulse. No such repetitive firing was seen. Barbiturates are believed to depress tissue cholinesterase activity only when administered over prolonged periods (see Augustinsson, 1948). Thus it seems unlikely that the effects which thiopentone can exert upon rat skeletal muscle operate by way of an anticholinesterase mechanism.

The present work shows that thiopentone decreases the voltage of the muscle action potential, increases its latency and prolongs its duration. These effects could occur if the rate of propagation of the potential was slowed, or if the wave of depolarization was wider, thereby taking longer to pass across the point from which the electrical record was made. Within the limits of the technique used here it cannot be decided which of these two possibilities operates.

However, there is evidence to show that agencies which slow the rate of propagation of the wave of depolarization along the muscle fibre membrane are associated with an augmentation of the twitch tension. For example, this is so at subnormal temperatures, and during the post-tetanic augmentation of the muscle twitch (Brown and von Euler, 1938). These authors found that the injection of potassium chloride could augment the twitch tension, prolong the muscle action potential and, in addition, depress the peak voltages. Similar changes have been found with quinine by Harvey (1939). More recently, the action of adrenaline in augmenting twitch tension in the isolated rat phrenic nerve-diaphragm preparation has been shown to be associated with similar electrical changes (Brown, Bülbring and Burns, 1948). These authors thought that, while temporal dispersion of the contractions of the individual fibres might play a part in producing the augmented tension with adrenaline, its contribution was not large.

Increase in the twitch tension, such as that illustrated in Fig. 1 with thiopentone, is consistent with a slower propagation of the muscle action potential associated with a slower and more prolonged contraction. In this connexion it may be remembered that in a simple muscle twitch there is insufficient time, before relaxation has set in, for the full tension of the muscle to be developed (Hill, 1949).

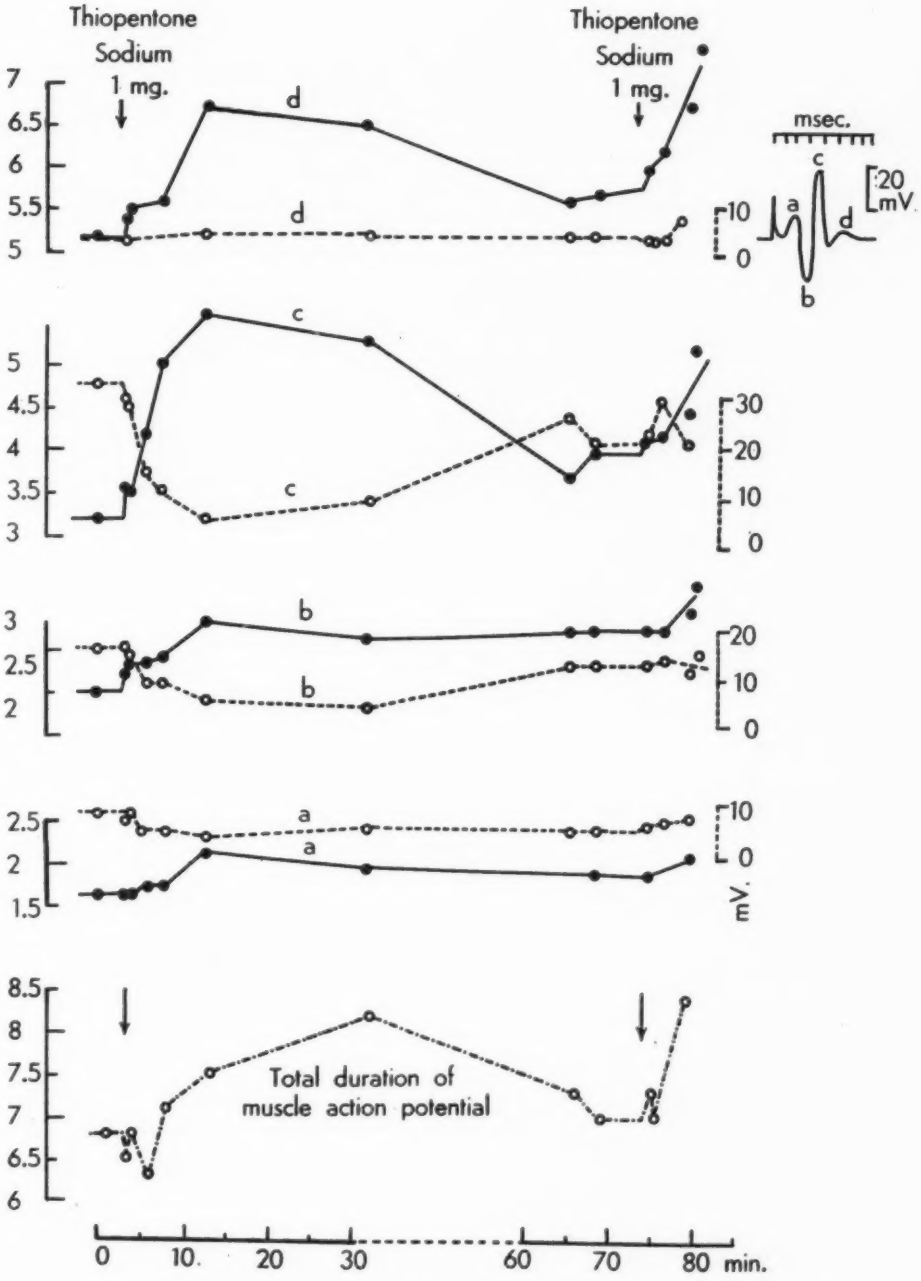


FIG. 6.—Graphical record of the experiment illustrated in Fig. 5. The open circles represent the voltage and the solid circles the latencies of the four peaks *a*, *b*, *c*, and *d* of the action potential record. Right-hand ordinates, amplitude in mV. Left-hand ordinates, latency in msec.

Little is known about the precise mode of action of barbiturates upon tissues. In two instances, however, respiratory enzyme systems are implicated —in the depression of respiration of rat brain slices *in vitro* (Quastel, 1943; Ghosh and Quastel, 1954), and in the depression of the oxygen consumption, associated with repetitive activity, in the rabbit's isolated superior cervical ganglion (Larrabee, Ramos, and Bülbring, 1950). But whether thiopentone sodium acts on skeletal muscle by affecting a barbiturate-sensitive stage of a respiratory enzyme remains to be determined.

## Summary

1. Thiopentone sodium, injected intra-arterially into the gastrocnemius-soleus muscle of a rat anaesthetized with urethane, augmented the twitch of the muscle when stimulated indirectly. This effect was similar to that seen in the rat obturator nerve anterior gracilis muscle preparation and in isolated frog or rat nerve-muscle preparations.

2. The intra-arterial injection of thiopentone sodium was associated with a decrease in voltage, an increase in latency and a prolongation of duration of the muscle action potential recorded from the surface of the anterior gracilis muscle of the anaesthetized rat.

3. The significance of these electrical changes in relation to the mechanical response of the muscle is discussed.


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*Brit. J. Pharmacol.* (1955), **10**, 147.

# ACTION OF PROGUANIL AND ITS METABOLITE ON NEUROMUSCULAR AND SYNAPTIC TRANSMISSION

BY

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(RECEIVED APRIL 6, 1954)

The toxicity of the antimalarial drug proguanil (" Paludrine ") varies with its route of administration, but it is relatively low (Curd, Davey and Rose, 1945). Sometimes death ensues rapidly, sometimes after a few days (Butler, Davey, and Spinks, 1947). Such delayed toxicity has been attributed to its effect on cholinesterase (pseudocholinesterase of the blood serum, benzoylcholinesterase of the guinea-pig liver: Blaschko, Chou and Wajda, 1947) and is increased by neostigmine (Vane, 1949). Vane has shown that proguanil competes with acetylcholine, blocking its action on isolated rabbit auricle and guinea-pig ileum, inhibiting transmission in the phrenic-diaphragm preparation of the rat and in the sciatic-gastrocnemius preparation of the cat. In the rectus abdominis of the frog small doses of proguanil enhanced the actions of acetylcholine, large doses decreased the response, and still larger doses provoked a contracture. Hawking and Perry (1948) found that proguanil only affected *Plasmodium, in vitro,* in the presence of liver slices, suggesting that an active metabolite has to be formed. Crowter and Levi (1953) showed that *in vivo* the antimalarial action is due to 4:6-diamino-1-*para*chlorophenyl - 1:2 - dihydro - 2:2 - dimethyl-1:3:5-triazine—hereafter called triazine.

It seemed desirable to investigate further the actions of proguanil and triazine on neuromuscular transmission, and this paper describes such experiments in cats, dogs and rats. The actions of proguanil on the frog rectus abdominis, and on synaptic transmission in the superior cervical ganglion of the cat, have also been investigated.

## METHODS

For the studies on neuromuscular transmission, cats and rats were anaesthetized with allobarbitone (Dial) and dogs with chloralose. The contractions of the tibialis anterior and the soleus in cats were induced by stimulation of the sciatic nerve, with ten supramaximal stimuli per minute (pulse duration, 1.0 msec.), from a Copeland stimulator (1951). In dogs, the movements of tibialis anterior and gastrocnemius were recorded; in rats, only the gastrocnemius. For observations on synaptic transmission in cats the cervical sympathetic was separated from the vagus and ligated below the point of stimulation. The contractions of the nictitating membrane were recorded in the usual way in response to supramaximal stimuli at 50 or 100/min. (pulse duration, 1.0 msec.) during which the drugs were usually injected intravenously. Sometimes the drugs were injected into the common carotid artery, all branches of which had been tied off except those supplying the superior cervical ganglion.

Arterial blood pressure was recorded in the cat and dog as a help in assessing the animal's condition. Artificial respiration was employed only in respiratory failure.

## RESULTS

### *Proguanil*

*Action on Neuromuscular Transmission.*—Intravenous injections of proguanil (5 to 10 mg./kg.) inhibit neuromuscular transmission in the cat. The inhibitions are more marked in soleus than in tibialis, and increase in intensity and duration on repetition of the injections (Fig. 1). Similar effects are found at the same dose-level in the dog and rat. Recovery from the block is delayed by decamethonium iodide (10 μg./kg.) and by eserine (400 μg./kg.). Where the decamethonium precedes the proguanil, the proguanil first increases the block, but thereafter there is a rapid restoration of transmission (Fig. 2). If the block is first developed under tubocurarine proguanil intensifies it and there is no such rapid recovery of transmission (Fig. 3, lower record). Responses to proguanil appear enhanced where transmission has recovered from block by either decamethonium (Fig. 3, upper record) or tubocurarine (Fig. 3, lower record), but these effects are difficult to evaluate because of the sensitizing or cumulative effects already referred to. When proguanil is given first, subsequent responses to decamethonium appear to be reduced and those to tubocurarine increased.

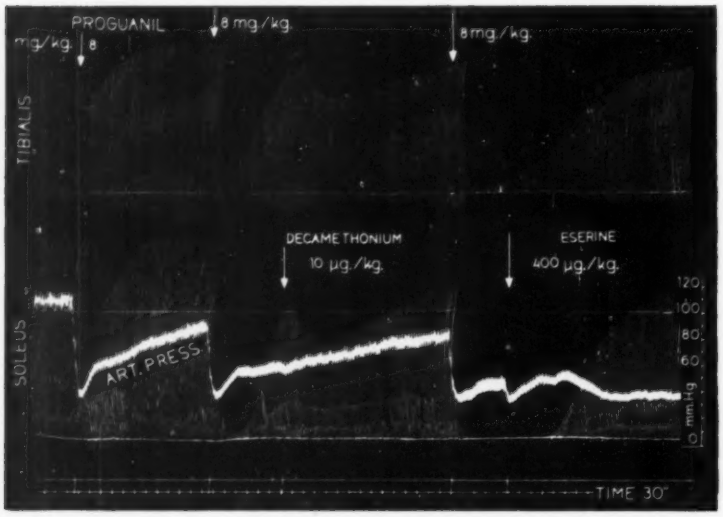


FIG. 1.—Cat (allobarbitone). Simultaneous records of contractions of the tibialis anterior and soleus muscle in response to single maximal shocks at 10/min. to the sciatic nerve. Record of blood pressure. From left to right, at arrows, i.v. 8 mg./kg. proguanil (twice); 10 µg./kg. decamethonium iodide; 8 mg./kg. proguanil; 400 µg./kg. eserine. Shows block of neuromuscular transmission by proguanil, and the delay in recovery produced by decamethonium and by eserine.

Although no evidence of a depolarizing action of proguanil on the synapses was found, these experiments gave some further evidence that proguanil and its metabolite triazine could act as competitive inhibitors. While the effect on the nictitating membrane appears to be brief, the responses to subsequent stimuli are diminished, reaching a minimum in some twenty minutes (Fig. 5B). Fig. 5C shows that faradization of the sympathetic chain (15 stim./sec.) rapidly restores normal transmission, and thereafter a further injection of proguanil has a two-stage action on the membrane. The usual initial block is followed, after partial recovery, by a more prolonged inhibition. This prolonged and delayed effect can be explained by the change in the molecule into its more active metabolite triazine. The biphasic character of the response does not appear as far as the blood pressure is concerned because the hypotensive action of

Because of the transitory nature of proguanil block, it is difficult to be dogmatic about its modification by anticholinesterases. In Fig. 1 eserine appears to prolong a block rather than diminish it, yet in other experiments eserine has appeared to reduce the cumulative effects of proguanil. In the cat, neostigmine is no better as an antagonist than eserine, but in the rat, as in Fig. 3, two injections of neostigmine relieve blocking, as does adrenaline.

*Action on Synaptic Transmission.*—The enhancement of a decamethonium block which precedes proguanil antagonism suggests that this antimalarial has a depolarizing as well as a curarimimetic action; but no evidence to support this has been obtained from a study of its actions on sympathetic synapses. In Fig. 4A the effect of increasing doses of proguanil on contractions produced by 50 stim./min. is shown between reference doses of ACh and adrenaline and stimulation at various frequencies (see legend). The proguanil response increases with the dose and is purely depressant, 5 mg./kg. producing a 60% inhibition. The sensitivities of the sympathetic synapse and the neuromuscular end plate would seem to be of the same order. Fig. 4B shows similar inhibitions of synaptic transmission with intra-ganglionic injections of proguanil. Here 50 µg. has almost as much effect as 1 mg./kg. i.v., and 250 µg. produces a very pronounced though brief inhibition.

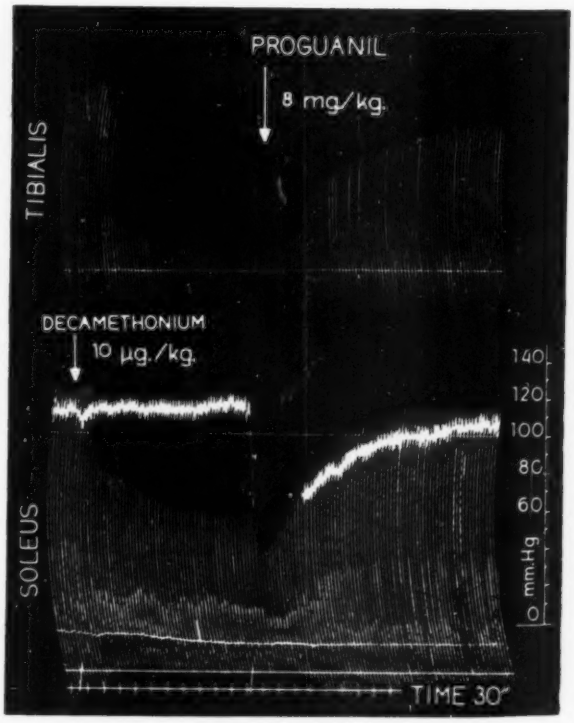


FIG. 2.—Similar preparation to that in Fig. 1. From left to right, at arrows, 10 µg./kg. decamethonium iodide; 8 mg./kg. proguanil. Shows that proguanil after decamethonium first increases block and then promotes restoration of neuromuscular transmission.

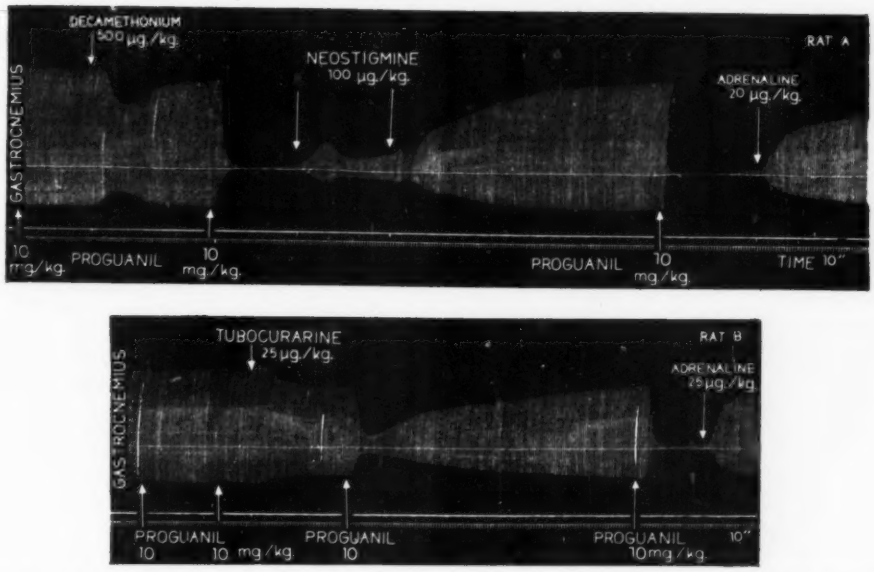


FIG. 3.—Rats (allobarbitone). Contractions of gastrocnemius induced by stimulation of the sciatic nerve (single maximal shocks at 10/min.). From left to right: upper record (rat A), 10 mg./kg. proguanil; 500 µg./kg. decamethonium iodide; 10 mg./kg. proguanil; 100 µg./kg. neostigmine (twice); 10 mg./kg. proguanil; 20 µg./kg. adrenaline. Lower record (rat B), 10 mg./kg. proguanil (twice); 25 µg./kg. tubocurarine; 10 mg./kg. proguanil (twice); 25 µg./kg. adrenaline. Shows enhancement of proguanil blocking action after recovery from decamethonium (upper record) and from tubocurarine (lower record) block.

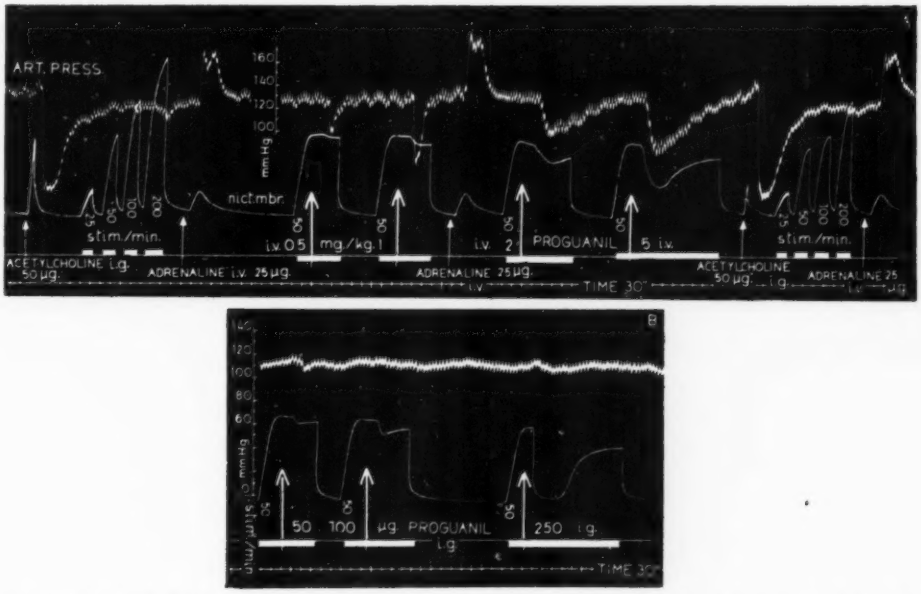


FIG. 4.—Cat (allobarbitone). Simultaneous records of blood pressure and nictitating membrane. From left to right: A (upper tracing), intra-ganglionic (i.g.) injection of 50 µg. acetylcholine; stimulations of the cervical sympathetic nerve (at 25, 50, 100, and 200/min.); i.v. injection of 25 µg. adrenaline. During successive stimulations at 50/min., 0.5, 1.0, 2, and 5 mg./kg. proguanil i.v. Between 2nd and 3rd proguanil injections, 25 µg. adrenaline i.v. Acetylcholine 50 µg. i.g. Stimulations of cervical sympathetic; adrenaline 25 µg. i.v. B (lower record), successive i.g. injections of 50, 100, and 250 µg. proguanil during stimulation of cervical sympathetic. Shows that proguanil blocks synaptic transmission both by intravenous (i.v.) and intra-ganglionic (i.g.) injection. This compound does not modify the adrenaline effect but lowers the stimulating action of ACh.

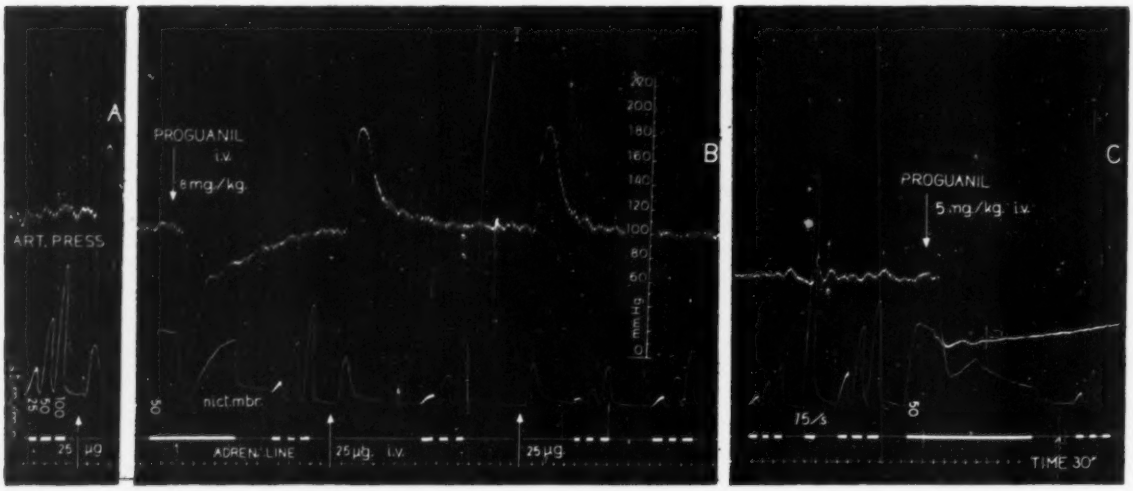


FIG. 5.—Cat (allobarbitone). Simultaneous record of blood pressure and nictitating membrane. Intravenous injections only. A—normal reactions of the cat: cervical sympathetic stimulations at 25, 50, and 100/min. respectively; 25 µg. adrenaline at arrow. B—during stimulation of cervical sympathetic (50/min.), 8 mg./kg. proguanil: a series of stimulations and of adrenaline injections (25 µg.). C—stimulations of the cervical sympathetic; faradization of the chain (15/sec.); stimulations of the chain. During stimulation at 50/min., 5 mg./kg. proguanil. Stimulations of the chain. Shows that proguanil action on synaptic transmission seems to be biphasic: a first transitory and short action is followed by a much longer inhibition. Faradization of the sympathetic chain restores transmission.

proguanil is much stronger than that of its metabolite.

*Triazine*

Qualitatively triazine acts as proguanil does, but quantitatively both neuromuscular and synaptic transmission are more affected by the metabolite: it is also more cumulative, for after repeated intravenous injections the response to 100 µg./kg. may equal the original response to 5 mg./kg.

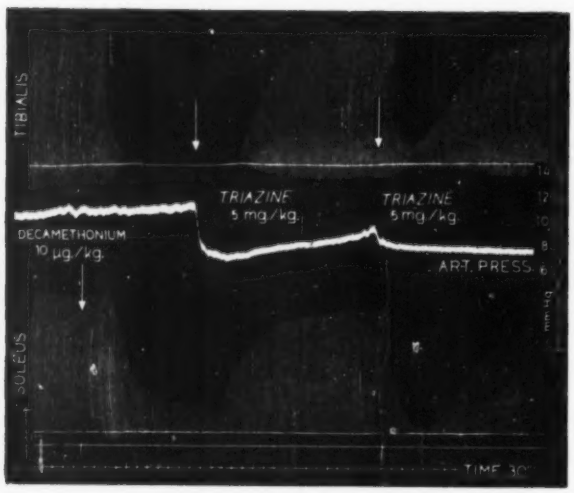


FIG. 6.—Similar preparation to that of Fig. 1. From left to right: 10 µg./kg. decamethonium iodide; 5 mg./kg. triazine (twice). Shows that triazine antagonizes blocking action of decamethonium.

Fig. 6 demonstrates its striking antagonism of decamethonium, while Fig. 7A and 7B give evidence of its effects on the synapse with one twentieth to one tenth of the dose required with proguanil. Neither when given intravenously, as in Fig. 7A, nor when injected into the ganglion through the ligated carotid, is there any apparent modification by triazine of the response of the nictitating membrane to adrenaline.

*Rectus Abdominis of Frog.*—Vane's observations (1949) on the actions of proguanil on the rectus muscle have been confirmed. This compound increases the action of ACh in low concentrations ($<10^{-6}$), but inhibits contraction at $10^{-5}$ and induces a contracture at $10^{-4}$. The frog rectus, however, unlike mammalian tissues, is less sensitive to triazine than to proguanil. Triazine, $10^{-5}$, inhibits the action of ACh $10^{-6}$ but not $10^{-5}$. With ACh $10^{-5}$ plus triazine $2 \times 10^{-4}$, the muscle first contracts then relaxes and is insensitive to a new dose of ACh $10^{-5}$, but rapidly becomes sensitive after washing. Even $2 \times 10^{-4}$ triazine does not produce such contractures as were found with proguanil.

## DISCUSSION

The evidence for competitive inhibition of neuromuscular transmission by proguanil and its metabolite is substantial. Thus their inhibitory action is not preceded by any increased muscle tonus, or increased response, as is usual with

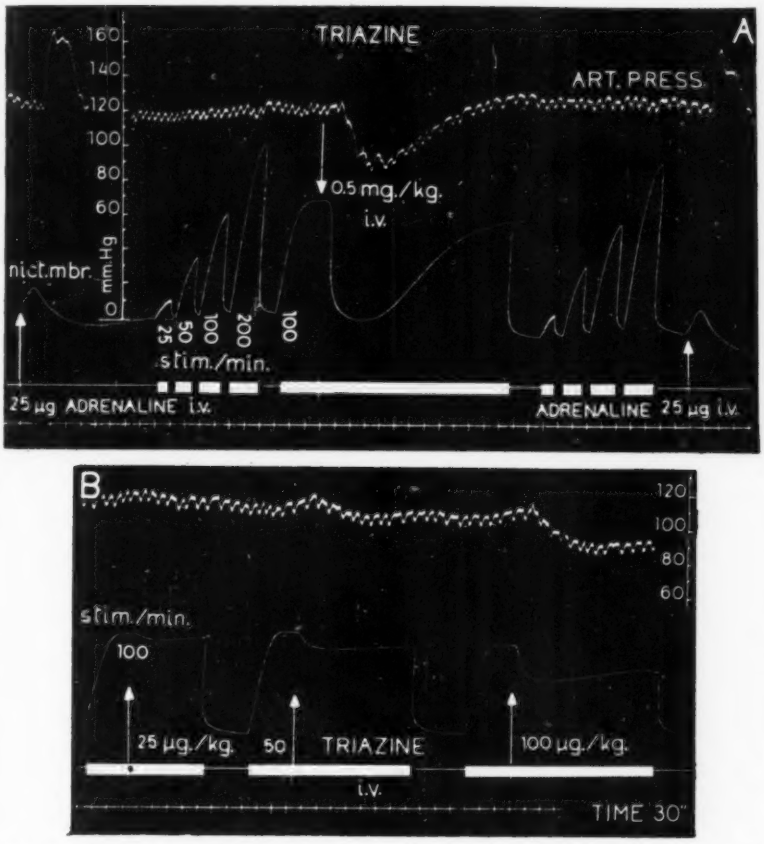


FIG. 7.—Similar preparation to that of Fig. 5. A—from left to right: 25 µg. adrenaline, stimulations of the cervical sympathetic chain (25, 50, 100, 200/min. maximal single shocks). During the stimulation (100/min.), intravenous injection of 0.5 mg./kg. triazine. Stimulations of the chain. 25 µg. adrenaline. B—triazine (25, 50, and 100 µg./kg. i.v.) during cervical sympathetic stimulation (100/min.). Shows that small doses of triazine (i.v.) inhibit synaptic transmission. Compare the doses with Fig. 4A.

decamethonium and other depolarizing blocking agents; they antagonize decamethonium and enhance the response to tubocurarine; their own blocking actions are reduced by adrenaline and by neostigmine; they are more active on soleus than on tibialis, and act at about the same dose level in cat, dog and rat. Eserine antagonism is, however, very slight if it exists, and the transitory additive effects of these substances on decamethonium block of neuromuscular transmission are difficult to interpret. No evidence of stimulation before the blocking of synaptic transmission has been found, but this does not rule out some possible depolarizing action on the motor end plate. It seems unlikely that the decamethonium enhancement is due to histamine release, for triazine gives no such depression of blood pressure as is seen with proguanil; moreover the recognized histamine-liberators seem to antagonize rather than to sum with decamethonium (Dallemagne and Philippot, 1953). It seems more reasonable to assume that it is the competitive inhibitory component of the action of decamethonium (Zaimis, 1952) that is enhanced by proguanil and triazine; this is the more likely since the additive effects are more marked on soleus than tibialis, soleus being more affected by competitive blocking drugs. Thus the actions of proguanil and triazine on the motor end plate are comparable to those of the higher homologues of the alkyl-trimethyl ammonium salts (Dallemagne and Philippot, 1951).

Triazine is somewhat more efficient than proguanil in blocking the motor end plate, and markedly more so on synaptic transmission. If, as has been suggested, the prolonged biphasic response of the nictitating membrane recorded in Fig. 5C is due to the conversion of proguanil to triazine, the cyclization of the biguanide chain must take place very rapidly in the body.

These results make it difficult to explain Vane's (1949) observations on the enhancement of the toxicity of proguanil by neostigmine—as an antagonist at the neuromuscular junction neostigmine might be expected to reduce the toxicity of proguanil—but this toxicity may not be due to its depression of neuromuscular transmission and hence of respiration.

## SUMMARY

1. Proguanil and its metabolite, triazine, are competitive inhibitors of neuromuscular and synaptic transmission.

2. The metabolite is more active than proguanil in inhibiting neuromuscular transmission and much more active in inhibiting synaptic transmission.

3. Proguanil is rapidly transformed into triazine in the organism.

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# EXPERIMENTAL CHOLERA IN INFANT RABBITS: A METHOD FOR CHEMOTHERAPEUTIC INVESTIGATION

BY

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Laboratory investigations on the chemotherapy of cholera have been handicapped by the absence of a suitable animal in which a disease resembling the human disorder could be reproduced. Sulphathiazole, sulphadiazine, sulphaguanidine (Griffitts, 1942a), chloramphenicol (Gauld, Schlingman, Jackson, Manning, Batson, and Campbell, 1949) and oxytetracycline (Terramycin) (Olejnik and Davidovitch, 1951) were shown to be effective in the treatment of mice experimentally inoculated with *Vibrio cholerae*. In all this work mice were inoculated intraperitoneally, and died with massive bacteraemia. Collier, Hall, and Waterhouse (1949) have developed the "mouse faecal suspension test" for evaluation of remedies for cholera, but the authors admit that "there can be little direct evidence that any laboratory tests provide reliable indication of clinical usefulness."

Several workers gave the vibrios to laboratory animals orally, but the results were not sufficiently consistent to have any practical application. Sabolotny (1894) demonstrated the susceptibility of *Spermophilus guttatus* to cholera vibrios given by mouth. Only half of the animals became infected; diarrhoea did not always occur and septicaemia was common. Metchnikoff (1894) could not induce the infection in kittens, puppies, mice, or gerbils. Diarrhoea and death were produced in 25 to 50% of suckling rabbits, but the results were irregular and unpredictable. Similar results were obtained by Sanarelli (1921), and Crendiropoulo (1921) showed that *V. cholerae* was destroyed by gastric juice. Klemperer (1894) tried unsuccessfully to produce cholera in rabbits by intra-intestinal inoculation. An unsuccessful attempt was also made to produce cholera in monkeys by damaging the intestinal wall with warm water or deep *x*-ray, followed by intra-intestinal inoculation of vibrios (Report, Scientific Advisory Board, 1946). Griffitts (1942b) demonstrated that the virulence of *V. cholerae* for mice was enhanced if the organisms were injected together with mucin.

The aim of the present work was to develop a method by which cholera could be produced readily in a laboratory animal, and to use it for the evaluation of anti-cholera drugs. If the results of study on experimental animals were to run parallel with the clinical findings with known drugs, the method would provide a means for further research on cholera. Our attempts to infect young mice, rats, and guinea-pigs by intra-intestinal inoculation of *V. cholerae* did not meet with success, but it was found that infant rabbits under certain conditions were highly susceptible.

## METHODS

*Experimental Animals.*—Infant rabbits, ten days old, of either sex, weighing 100–200 g. were used. The young ones live on their mothers' milk and do not generally open their eyes before the 10th or the 11th day. The litters were kept with their respective mothers in separate cages.

*Preparation of Infective Dose.*—A strain of *V. cholerae* (Inaba 569 B), maintained at this Institute for preparation of vaccine, was used. The freeze-dried culture was regenerated in broth, plated on agar slants, and incubated overnight. A 1 mm. loopful of the culture was suspended in 10 ml. of nutrient broth and again incubated for 3 hr. At the end of this period the broth contained approximately 1,000 million vibrios per ml. This was suitably diluted before use. The viable cholera vibrios were counted by the technique of Sokhey (1939) described for plague organisms.

*Evolution of a Virulent Strain (Table I).*—When infant rabbits were fed with 100 million vibrios per 100 g. body weight, only 1 of 8 suffered from severe diarrhoea, and collapsed after 48 hr. In the remaining animals *V. cholerae* failed to cause the infection. When 100 million organisms per 100 g. body weight were injected directly into the small intestines of infant rabbits under ether anaesthesia, the mortality

was increased. Of 4 animals in the first expt. 2 died within 72 hr. with severe diarrhoea; watery stools were not observed in the remaining 2. Of the 4 rabbits of the second group, 3 had liquid motions. Two of them recovered, while the third became emaciated and died after 4 days. One animal from this group was completely resistant.

TABLE I

INFECTION OF INFANT RABBITS WITH *V. CHOLERAE* (INABA)

| Strain | Infective Dose/100 g. Body Wt. | No. with Diarrhoea/ No. Infected | No. Dead/No. Infected |
|---|---|---|---|
| Unpassaged, without mucin .. | $100 \times 10^6$ (oral) | 1/8 | 1/8 |
| ,, ,, | $100 \times 10^6$ (i.i.*) | 5/8 | 3/8 |
| Unpassaged, with 5% mucin .. .. | $50 \times 10^6$ ,, | 7/25 | 11/25 |
| Passaged, with 5% mucin .. .. | $50 \times 10^6$ ,, | 12/12 | 12/12 |
| Passaged, without mucin .. | $50 \times 10^6$ ,, | 2/2 | 2/2 |
| ,, ,, | $10^4$ ,, | 86/86 | 86/86 |
| ,, ,, | $10^3$ ,, | 11/11 | 11/11 |

* i.i. = intra-intestinal.

When 50 million vibrios were injected into the intestine together with mucin, 11 out of 25 died. Of the dead ones only 7 animals had diarrhoea. Vibrios were isolated from heart blood shortly after death in three animals. The strain was preserved by freeze-drying.

The strain was regenerated as described above and infant rabbits were infected intra-intestinally with varying numbers of vibrios. Mucin was not used, but 86 animals which received 10,000 vibrios per 100 g. body weight all showed signs of a disease resembling human cholera in many respects. The virulent strain isolated from heart blood was used in all subsequent experiments. Virulence was maintained by animal passage every 6 months.

Another strain of *V. cholerae* (Ogawa 41), taken from our stock cultures, when tested on infant rabbits behaved in the same way as Inaba 569 B.

*Biochemical Studies.*—Measurements were made of the cell volume, urea, and non-protein nitrogen contents of samples of oxalated heart blood obtained from infected animals. Specimens were not collected until life, in our estimation, could not have been possible for more than 2 hr. Owing to the difficulty of obtaining blood from dehydrated animals, most of the samples were pooled from 2 animals. In normal rabbits each sample was obtained from a single animal.

*Susceptibility of Rabbits of Various Ages.*—Infant rabbits were infected intra-intestinally with a heavy dose of 100 million vibrios per 100 g. body weight. Animals less than 10 days old were not sufficiently mature to withstand the injurious effect of anaesthetic ether, and therefore were not used.

*Habitat of the Vibrios.*—For this experiment groups of infected rabbits were killed at varying intervals 8–49 hr. following inoculation. From each animal 0.25 ml. of heart blood and a portion of the liver were cultured in peptone water for isolation of the vibrios. The liver was approached through the chest cavity and the diaphragm to avoid handling intestines.

*Drugs.*—For *in vitro* tests formo-sulphathiazole ("Formo-Cibazol," Ciba; formaldehyde sulphathiazole), sulphaguanidine, chloramphenicol, and chlortetracycline (aureomycin) hydrochloride were suspended in 6% aqueous gum acacia. The first three were sterilized at 10 lb./sq. in. for 10 min.; the chlortetracycline suspension was prepared aseptically. Oxytetracycline hydrochloride was dissolved in sterile aqueous solution of HCl (*p*H 2). For *in vivo* tests all the substances were suspended in 6% aqueous gum acacia and administered orally with a small glass pipette.

In vitro *Experiments.*—A serial dilution method was employed in which the total volume of the medium in each tube was kept constant (10 ml.) but the concentrations of the compounds were varied. The substances were tested in peptone water (*p*H 7.4) and in a protein-free casein hydrolysate medium (Sokhey, Habbu, and Bharucha, 1950). The potency of the drug was assessed against $10^3$, $10^6$, and $10^8$ vibrios per ml., obtained by suitably diluting a 3 hr. growth (rabbit-passaged strain of Inaba 569 B) of *V. cholerae*. A large inoculum was used in the belief that it might more nearly represent the condition in which the drugs were required to act in the body. The culture tubes were incubated at 37° C. for 24 hr.

*Testing of Drugs in Experimental Cholera*

*Standard Dose Schedule.*—Preliminary study suggested that a nine-dose treatment, one dose every 8 hr. for 3 days, was necessary to obtain the optimum effect of a drug. Chloramphenicol, chlortetracycline, and oxytetracycline hydrochlorides were administered in doses of 100 mg./kg. Sulphaguanidine and formo-sulphathiazole were given in amounts of 750 and 650 mg./kg. respectively. Those animals which recovered from the attack received the full regimen of 9 doses; otherwise the amounts of drug administered depended on the survival periods.

Treatment was begun 1 hr. before the infection, or 8, 16, or 24 hr. following it. The onset of diarrhoea in the infected rabbit was taken as the first definite sign of the beginning of the attack. The prophylactic and curative values of a drug were judged by the results obtained from beginning treatment before inoculation and 24 hr. after inoculation. For each experiment, a group of infected untreated animals was used as a control.

*Saline Treatment.*—Within two hours of the onset of diarrhoea in infant rabbits suffering from cholera, each animal received a dose of 100 mg./kg. of chlortetracycline hydrochloride followed by 2 ml. hypertonic saline (8 g. sodium chloride and 0.25 g. calcium chloride in 570 ml. water) and 1 ml. of alkaline saline (6 g. sodium chloride and 11 g. sodium bicarbonate

in 570 ml. water) intraperitoneally. It was not possible to give salines intravenously. The dose of chlortetracycline was repeated after 6 hr. and, later on, at intervals of 8 hr. Both hypertonic and alkaline salines were given every 3 hr. until each of the animals had received 12 ml. of fluid. Control animals did not receive either the drug or the salines.

## RESULTS

### *Experimental Cholera in Infant Rabbits*

*Signs and Symptoms.*—About 16–24 hr. following infection with 10,000 vibrios (passaged strain), the animals showed signs of illness. They became less active and huddled together. On an average the diarrhoea made its appearance 22 hr. after inoculation, but the onset was delayed up to 44 hr. in a few rabbits. The diarrhoea was mild at the beginning but became severe. The animals survived for a mean period of 32 hr. from the time the infective dose was given, or 10 hr. after the first appearance of the liquid motions. Death occurred as early as 22 hr. or as late as 52 hr. after infection. In some cases death took place 4 hr. after the diarrhoea began, whereas in a few instances a day elapsed before the animal died. During the terminal stage, the animal lay exhausted and did not like to move unless seriously disturbed. The skin became cold and the hair rough. Cramps in the muscles were occasionally noticed before death. Vomiting was never observed. *V. cholerae* was frequently isolated from the watery stools, which also contained lumps of mucus.

When rabbits were inoculated with 50 million vibrios/100 g. body weight, diarrhoea was observed 14 hr. after infection, and most died within 20 hr.

*Post-mortem Changes.*—The animal looked emaciated. The hair was rough and lustreless and the whole undersurface of the body was smeared with liquid discharges from the bowel. On opening the abdomen the distended large intestine stood out prominently. It was rather pale in appearance and full of watery fluid containing flakes of mucous membrane. The vibrios were recovered easily from the liquid content of the bowel. The stomach was always distended with coagulated milk. The small intestine was generally hyperaemic and in places was scarlet in colour. In some animals no congestion was noticed, except that a few vessels stood out prominently. The lower part of the ileum was distended but not so much as the upper part of the large intestine. The small intestine contained a thick viscid liquid, and parts of the mucous membrane had been destroyed. The liver, spleen and kidney were either normal in appearance or congested. The auricles contained thick dark blood and the ventricles were empty and contracted.

### *Biochemical Studies*

A summary of the biochemical findings is given in Table II. A significant degree of haemoconcentration was observed in infected rabbits. The average non-protein nitrogen and urea contents of the blood of rabbits suffering from cholera were significantly higher than normal.

TABLE II

BIOCHEMICAL CHANGES IN BLOOD OF INFANT RABBITS INFECTED INTRA-INTESTINALLY WITH 10,000 *V. CHOLERAE* (INABA) PER 100 G. BODY WEIGHT

| | Rabbits | No. of Animals | Range | Mean | t | Level of Significance |
|---|---|---|---|---|---|---|
| Blood cell volume % | Normal | 10 | 32–41 | 36·70 | 6·733 | 1% |
| | Infected | 10 | 38–52 | 47·60 | | |
| Plasma non-protein nitrogen mg./100 ml. | Normal | 11 | 29–60 | 42·73 | 4·433 | 1% |
| | Infected | 10 | 41–85 | 65·50 | | |
| Blood urea mg./100 ml. | Normal | 11 | 13–28 | 18·64 | 5·181 | 1% |
| | Infected | 13 | 22–53 | 37·07 | | |

### *Susceptibility of Rabbits of Different Ages*

The susceptibility of young rabbits to *V. cholerae* at different ages is shown in Table III. Up to the sixteenth day of life, the animals remained highly susceptible. Beyond this period the rabbits progressively resisted the infection until they were a month old, when it became impossible to infect them. As the susceptibility lessened, the incidence of diarrhoea decreased. Post-mortem changes in all of the rabbits were suggestive of the infection. Susceptibility was not related to body weight, nor to seasonal variations.

TABLE III

SUSCEPTIBILITY OF YOUNG RABBITS TO *V. CHOLERAE* (INABA) WHEN INJECTED INTRA-INTESTINALLY WITH 100 MILLION ORGANISMS PER 100 G. BODY WEIGHT

| Age of Animals (Days) | Symptoms of Cholera | Mortality |
|---|---|---|
| 16 | 6/6 | 6/6 |
| 21 | 3/5 | 4/5 |
| 31 | 0/5 | 0/5 |
| 45 | 0/2 | 0/2 |

### *Habitat of the Vibrio*

Specimens of blood and liver of 50 rabbits were examined. Vibrios were recovered from the blood of 3 animals 24–29 hr. after in-

TABLE IV

ACTION *IN VITRO* OF CHLORAMPHENICOL, CHLORTETRACYCLINE, OXYTETRACYCLINE, FORMO-SULPHATHIAZOLE AND SULPHAGUANIDINE AGAINST *V. CHOLERAE* (INABA)

| Drug | Casein Hydrolysate Medium | | | | Peptone Water Medium |
|---|---|---|---|---|---|
| | Vibriostatic Concn. ($\mu$g. ml.) Against $10^6$ Vibrios ml. | Vibriocidal Concn. ($\mu$g./ml.) Against $10^3$ Vibrios/ml. | $10^6$ Vibrios ml. | $10^8$ Vibrios/ml. | Vibriocidal Concn. ($\mu$g./ml.) Against $10^6$ Vibrios/ml. |
| Chloramphenicol | 2·5 | 2·0 | 5·0 | 100·0 | 2·5 |
| Chlortetracycline hydrochloride | 50·0 | 10·0 | 100·0 | 100·0 | 100·0 |
| Oxytetracycline hydrochloride | 10·0 | 10·0 | 50·0 | 250·0 | 25·0 |
| Formo-sulphathiazole | 250·0 | 20·0 | — | — | — |
| Sulphaguanidine | >2,000·0 (No inhibition) | >2,000·0 (No inhibition) | — | — | — |

fection, and from the liver of 1 animal which did not show a positive blood culture. All the rabbits which showed positive blood or liver cultures suffered from diarrhoea, but there were many other animals with diarrhoea in which bacteraemia or the presence of vibrios in the liver could not be demonstrated.

### In vitro *Experiments*

The results are given in Table IV. In casein hydrolysate medium, formo-sulphathiazole and sulphaguanidine were ineffective even when the inoculum was small. The antibiotics were active in both types of medium against large and small inocula. A synthetic compound, $\alpha$-bromo-*p*-nitrocinnamic aldehyde, reported to be highly bactericidal against a varied set of organisms (Affonso and Khorana, 1952), was found to be vibriocidal in a concentration of 20 $\mu$g./ml. against 100 million organisms in casein hydrolysate medium. The compound is insoluble in water and sparingly soluble in common solvents and also possesses low oral toxicity in white mice. Unfortunately, the substance is too toxic for the infant rabbit.

TABLE V

THE ACTION OF ORALLY ADMINISTERED VIBRIOCIDAL DRUGS ON INFANT RABBITS INFECTED INTRAINTESTINALLY WITH 10,000 *V. CHOLERAE* (INABA) PER 100 G. BODY WEIGHT

| Treatment Started (Hr. Before or After Infection) | Treated Infected Rabbits: Mean Time of Onset of Diarrhoea (hr.) | Mean Survival Time (hr.) | Mortality* | Untreated Infected Rabbits (Controls): Mean Time of Onset of Diarrhoea (hr.) | Mean Survival Time (hr.) | Mortality* |
|---|---|---|---|---|---|---|
| *Chloramphenicol* (Dose 100 mg./kg.) | | | | | | |
| 1 hr. before | Nil | — | 0/7 | 21·0 | 31·5 | 4/4 |
| 8 „ after | „ | — | 0/6 | 23·3 | 31·2 | 4/4 |
| 16 „ „ | 30·0 | 39·0 | 3/12 | 21·8 | 31·8 | 8/8 |
| 24 „ „ | 22·6 | 31·3 | 9/9 | 20·5 | 28·0 | 8/8 |
| *Oxytetracycline Hydrochloride* (Dose 100 mg. kg.) | | | | | | |
| 8 hr. after | Nil | — | 0/7 | 21·3 | 29·8 | 4/4 |
| 16 „ „ | 23·3 | 37·3 | 3/12 | 23·0 | 33·5 | 4/4 |
| 24 „ „ | 22·0 | 38·0 | 6/6 | 21·0 | 30·0 | 4/4 |
| *Chlortetracycline Hydrochloride* (Dose 100 mg./kg.) | | | | | | |
| 8 hr. after | Nil | — | 0/6 | 21·0 | 32·0 | 4/4 |
| 16 „ „ | 26·5 | 42·3 | 4/12 | 22·3 | 33·5 | 8/8 |
| 24 „ „ | 22·0 | 32·0 | 6/6 | 23·5 | 36·0 | 4/4 |
| *Sulphaguanidine* (Dose 750 mg./kg.) | | | | | | |
| 1 hr. before | Nil | — | 0/5 | 28·0 | 34·0 | 1/1 |
| 8 „ after | 24·3 | 32·0 | 5/9 | 24·6 | 33·3 | 5/5 |
| 16 „ „ | 26·0 | 33·3 | 4/4 | 26·0 | 34·0 | 2/2 |
| *Formo-sulphathiazole* (Dose 650 mg./kg.) | | | | | | |
| 1 hr. before | 36·0 | — | 0/7 | 19·5 | 24·5 | 4/4 |
| 8 „ after | 25·0 | 30·3 | 8/8 | 20·0 | 26·3 | 6/6 |
| *Chlortetracycline Hydrochloride* (Dose 100 mg./kg.) *and Salines* | | | | | | |
| After onset of diarrhoea | 23·3 | 36·9 | 9/9 | 28·0 | 33·2 | 4/4 |

* Ratio of animals dead to animals infected.

*Testing of Drugs in Experimental Cholera*

Table V summarizes the effect of the 5 compounds tried in experimental cholera. Formosulphathiazole prevented death only when administration of the drug was begun 1 hr. before the animals were infected.

When the drugs were administered 8 hr. after the infection, the three antibiotics prevented all symptoms of the disease from appearing. Sulphaguanidine had some activity, but formo-sulphathiazole was quite ineffective.

When treatment was begun 16 hr. after infection, sulphaguanidine failed to effect any improvement, but the three antibiotics were of about equal potency in combating the infection. At the beginning of treatment diarrhoea had not yet appeared. It is interesting to record that 2 or 3 rabbits from each group treated with antibiotics later developed diarrhoea, but they ultimately recovered completely under the treatment. The treated animals which died continued to pass watery stools and at autopsy the large intestine was full of fluid. Congestion of the vessels of the small intestine was not seen as frequently as in the controls.

Diarrhoea had already begun in animals treated 24 hr. after infection. Under these conditions none of the antibiotics had any effect upon the course of the illness and all the animals died before the fourth dose could be administered.

The treatment of rabbits with salines and chlortetracycline did not bring about any improvement in survival time or mortality rate.

## Discussion

The results of the study show that experimental cholera in the rabbit closely resembles the human disorder. The rapid onset of the disease and its short duration, profuse diarrhoea, dehydration, congestion of the small intestine and desquamation of the intestinal epithelium are common to both. Moreover, there is a significant increase of the non-protein nitrogen, urea and cell volume of the blood of rabbits suffering from cholera. In clinical cases too, appreciable increase in the cell volume (Pasricha and Malik, 1940 ; Taylor, 1941), plasma non-protein nitrogen (Shorten, 1918 ; Dhar, Dhar, and Adhyee, 1930 ; Banerjee, 1936 ; Pasricha and Malik, 1940 ; Chatterjee, 1941) and blood urea (Shorten, 1918 ; Banerjee, 1936 ; Pasricha and Malik, 1940 ; Chatterjee, 1941) is observed. These findings are not so definite as in rabbit cholera—perhaps because the mortality rate of the patients was much lower than in the experimental animals.

There are several features in which the disease in the rabbit differs from cholera in man. For example, vomiting is never seen in the rabbit. Unlike rabbit cholera, the pathological picture is variable in clinical cases (Rogers, 1911). Marked congestion of the small intestine, and, to a less extent, of the large intestine was seen in only 30% of human cases (Chatterjee, 1939). Distension of the large intestine with fluid is characteristic of the animal disease. Such differences are not altogether unexpected in view of the differences of anatomy and physiology in the rabbit and man.

Controversy concerning the role of vibrios in producing septicaemia in cholera has arisen because of failure to isolate them from the circulation during the active stage of the disease. However, there is indirect evidence to show that the vibrios occasionally enter the general circulation. Greig (1913a) recovered vibrios from the urine of cholera patients and also showed their presence in the lung and kidney (Greig, 1913b). Pasricha, de Monte and Chatterjee (1938) isolated *V. cholerae* from a few drops of blood obtained by liver biopsy in a boy suffering from cholera. All attempts to isolate the vibrios from the circulation have so far failed (Greig, 1919 ; de Monte and Gupta, 1938). In the present study, *V. cholerae* remained localized within the intestine of the rabbit and only occasionally entered the blood stream or the tissues.

Adult rabbits were immune to cholera ; young ones were highly suceptible up to the 16th day of life. Sanarelli (1921) believes that the development of immunity is associated with the change of diet from mother's milk to green vegetables. Arnold and Shapiro (1930) believe that an alteration in the reaction of the contents of the upper part of the intestine from slightly acid to alkaline brings about the change in susceptibility.

The difficulties of clinical assessment of drugs in the treatment of cholera are shown by the contradictory statements made by different workers about the efficacy of the same drug. The problem is complex, because no really effective drug therapy is known, and the activity of a new drug can be measured only against the effect of saline administration. Sulphaguanidine is stated to be potent against cholera (Chopra, de Monte, Gupta, and Chatterjee, 1941 ; Pasricha, Paul, Das Gupta, and Das, 1947), and to show low toxicity at an effective dose. The beneficial effect is statistically significant (Gupta, Chatterjee, Paul, and Ghose, 1945 ; Chu, Huang, Chang, and Kao, 1946 ; and Seal, 1947). The effectiveness of the drug has been questioned by Carruthers (1942) and Lahiri (1948,

1951). Lahiri demonstrated that, although the drug seemed to produce a beneficial effect, the results in his patients were not statistically significant. Formo-sulphathiazole was also stated to be an effective drug (Bhatnagar, Fernandes, De Sa, and Divekar, 1948). Subsequent workers (Chaudhuri, Ghosal, and Rai Chaudhuri, 1950 ; Lahiri, 1951 ; Roy Chaudhuri, Chaudhury, and Chadha, 1952) could not confirm this. In experimental cholera, both sulphaguanidine and formo-sulphathiazole possess no curative value.

Clinical trials of chloramphenicol (Chaudhuri, Ghosal, Mondal, and Chakravarty, 1952 ; Roy Chaudhuri *et al.*, 1952), chlortetracycline (Roy Chaudhuri *et al.*, 1952 ; Seal, Ghosh, and Ghosal, 1954) and oxytetracycline (Das, Ghosal, Gupta, and Chaudhuri, 1951 ; Konar and Sen Gupta, 1951 ; Roy Chaudhuri *et al.*, 1952 ; Das, Ghosal, Gupta, and Mondal, 1953 ; Das, Ghosal, and Gupta, 1953) do not suggest that they are therapeutically effective. The results of experimental trials of these drugs in rabbit cholera confirm the clinical findings, but suggest that these remedies might prove valuable in preventing the disease in man. The low toxicity and ease of administration of the antibiotics would make them especially valuable for contact cases.

From the therapeutic point of view the results of the *in vitro* tests have little importance because all these drugs have proved to be of little value in experimental and clinical cholera. Nevertheless it seems that the vibriocidal power of the compounds runs parallel with their ability to render the stools of cholera patients free from the vibrio. It has been reported that chloramphenicol (Chaudhuri *et al.*, 1952 ; Roy Chaudhuri *et al.*, 1952), chlortetracycline (Roy Chaudhuri *et al.*, 1952), and oxytetracycline (Das *et al.*, 1953) quickly remove the vibrios from the stools of patients. Formo-sulphathiazole, a poor vibriocidal compound, exerts a slight beneficial effect (Lahiri, 1951 ; Roy Chaudhuri *et al.*, 1952). It is doubtful whether sulphaguanidine, which is devoid of vibriocidal power, causes disappearance of the vibrios from stools earlier than in the controls (Chu *et al.*, 1946 ; Lahiri, 1951).

Experimental evidence suggests that the great mass of vibrios is confined to the intestinal lumen, and toxic substances are formed there as a result of their growth. These are probably absorbed early in the disease and diarrhoea may be a manifestation of the toxaemic state. Such a condition calls for immediate neutralization of the circulating toxin and prevention of further growth of the vibrios in the intestine by oral administration of compounds which are powerful vibriocides as well as relatively non-absorbable. The role of antitoxic serum in the treatment of experimental cholera is under investigation.

## Summary

1. A method has been described for inducing cholera in infant rabbits which closely resembles the human disease.

2. The disease is produced in ten-day-old rabbits by infecting them intra-intestinally with animal-passaged strain of Inaba or Ogawa subtypes. It is characterized by severe diarrhoea, dehydration and occasional cramps in the muscles. A significant increase in the plasma non-protein nitrogen, blood urea and blood cell volume is observed.

3. Post-mortem examination shows congestion of the blood vessels of the small intestine, areas of desquamation of the epithelium, and distension of the large intestine with watery fluid containing flakes of mucous membrane.

4. The vibrios are generally localized within the intestine and only occasionally make their entry into the circulation.

5. Infant rabbits are highly susceptible to *V. cholera* up to the sixteenth day of life, but later progressively resist infection and are completely immune by the end of a month.

6. A method for evaluation of drugs against cholera is described. Chloramphenicol (" chloromycetin "), chlortetracycline (aureomycin) and oxytetracycline (" terramycin ") possess excellent protective action, but they lack curative property even when administered at the early stage of the disease. The preventive value of sulphaguanidine is less than that of the antibiotics, but is superior to formaldehyde sulphathiazole (formo-sulphathiazole, " Formo-Cibazol ").

7. The quantitative nature of the test is well demonstrated for the chemotherapeutic substances studied, and the results are in agreement with the reported clinical findings.

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# PROTECTION AGAINST ANAPHYLACTIC SHOCK BY VARIOUS SUBSTANCES

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In view of the close relationship between anaphylactic shock and allergic disease, it is not surprising that many substances that antagonize anaphylactic shock (antihistamines, aminophylline, atropine and many sympathomimetic amines—Feinberg, Malkiel, Bernstein, and Hargis, 1950; Herxheimer and Rosa, 1953—) are also of great therapeutic value in allergic disorders. Anti-anaphylactic action is, however, not confined to such substances. Claims that a number of other drugs, differing widely in composition, have such an effect have been critically reviewed by Hill and Martin (1932). Because of the potential therapeutic value of anti-anaphylactic drugs, it was decided to investigate a number of substances reviewed by them and also some others.

## METHODS

Anaphylactic shock was produced in guinea-pigs by the "microshock" method (Herxheimer, 1952) in which the time from exposure to an antigenic aerosol to convulsion point (preconvulsion time) is regarded as a measure of the severity of the shock. If the time required to develop shock is significantly prolonged under the influence of a drug, this drug is regarded as having a protective effect. The degree of protection is calculated from the expression $100\left(1-\frac{C}{T}\right)$, where C is the preconvulsion time in the control experiment and T the preconvulsion time under the influence of the drug (Armitage, Herxheimer and Rosa, 1952).

The substances investigated were (a) drugs with a hypnotic or sedative action—morphine, chloral hydrate, phenobarbitone, chlorpromazine, (b) potentially spasmolytic drugs—pethidine, khellin, "Buscopan" (hyoscine-*N*-butyl bromide; Wick, 1951), methantheline (2-diethylaminoethyl xanthen-9-carboxylate methobromide, "Banthine") and propantheline (2′-di*iso*propylaminoethyl xanthen-9-carboxylate methobromide, "Probanthine"), (c) various other substances—salicylic acid, phenylbutazone ("Butazolidin"), barium chloride, sodium cyanate, caffein, ascorbic acid, cyanocobalamin, phenylephrine, neosynephrine, dibenzyline, and heparin.

## RESULTS

The substances which had no anti-anaphylactic action are listed in Table I. Morphine and caffeine had a protective effect in high doses only (50 mg. and 100 mg./kg. respectively). Buscopan, methantheline, propantheline, pethidine, and sodium cyanate had an anti-anaphylactic effect (Table II).

TABLE I

SUBSTANCES SHOWING NO ANTI-ANAPHYLACTIC ACTION AT SPECIFIED DOSE-LEVELS

| Substance | Dose (mg./kg.) | Route of Admin. |
|---|---|---|
| Ascorbic acid | 10, 20 | i.m. |
| Barium chloride | 10 | i.p. |
| Chloral hydrate | 300 | ,, |
| Chlorpromazine | 3 | i.m. |
| Cyanocobalamin | 0·05, 1·0 | ,, |
| Dibenzyline | 5, 10 | ,, |
| Heparin | 5,000 (u./kg.) | i.p. |
| | 10,000 ,, | |
| | 20,000 ,, | |
| Khellin | 20, 40 | i.m. |
| Phenobarbitone | 1 | i.p. |
| Phenylbutazone | 50 | i.m. |
| Phenylephrine | 0·2, 0·5, 1·0 | ,, |
| Salicylic acid | 40 | ,, |

## DISCUSSION

Sedatives were included in this investigation because they are sometimes clinically effective in allergic disorders. The action of morphine seemed of particular interest, since its use in asthma is condemned by many because of its depressant action on the repiratory centre. It is also a histamine liberator (Feldberg and Paton, 1949; Nasmyth and Stewart, 1949). In the present experiments morphine did not protect against anaphylactic shock except in a very high dose. The absence of any such effect with lower doses makes it probable that it has no specific anti-anaphylactic action. The effect of this high dose is possibly due to an indirect toxic influence. The claim that chloral hydrate has an anti-anaphylactic action (Banzhaf and Famulener, 1908) has not been confirmed by the present experiments. It has been

TABLE II

PERCENTAGE PROTECTION AFFORDED BY VARIOUS DRUGS AGAINST MICROSHOCK

(n, No. of animals; *, significant protection)

| Drug | Dose (mg./kg.) | n | Mean ± S.E. |
|---|---|---|---|
| "Buscopan" .. | 0·01 | 4 | 10·5 ± 13·4 |
| | 0·1 | 7 | 43·0 ± 5·9* |
| | 0·5 | 6 | 31·3 ± 7·7* |
| | 1·0 | 5 | 48·6 ± 10·1* |
| | 5·0 | 7 | 62·0 ± 4·9* |
| | 20·0 | 7 | 66·3 ± 5·5* |
| | 50·0 | 5 | 37·0 ± 13·7 |
| | 80·0 | 7 | 76·7 ± 4·1* |
| Caffeine .. .. | 100 | 5 | 51·2 ± 7·0* |
| Methantheline .. | 2 | 4 | 27·0 ± 15·8 |
| | 5 | 7 | 59·6 ± 6·5* |
| | 10 | 6 | 63·0 ± 4·7* |
| | 25 | 5 | 69·2 ± 13·6* |
| Morphine .. .. | 50 | 4 | 51·2 ± 7·9* |
| Pethidine .. .. | 10 | 5 | 16·0 ± 5·8 |
| | 20 | 5 | 43·4 ± 9·6* |
| | 40 | 6 | 50·3 ± 7·2* |
| Propantheline .. | 0·25 | 5 | 14·6 ± 13·4 |
| | 0·5 | 6 | 41·2 ± 8·8 |
| | 1·0 | 5 | 65·2 ± 8·6* |
| | 10 | 6 | 73·8 ± 4·1* |
| | 20 | 6 | 62·8 ± 4·1* |
| Sodium cyanate .. | 50 | 3 | 65·0 ± 8·7* |
| | 100 | 7 | 59·7 ± 9·3* |
| | 200 | 4 | 78·0 ± 5·3* |

said that chlorpromazine, a near relative of promethazine, has no antihistaminic action and that it is a centrally active sedative. The present experiments confirm that, unlike promethazine, it has no anti-anaphylactic effect. In other, unpublished experiments, it was confirmed that it did not suppress histamine shock in the guinea-pig except in doses as high as 10 mg./kg.

Buscopan, methantheline and propantheline all have an atropine-like effect. As atropine has an anti-anaphylactic action, a similar effect of these substances might be expected. It is remarkable that high doses were tolerated by the animals without any sign of toxicity. Significant protection was present with very small doses (0.1 mg./kg. buscopan and 0.25 mg./kg. propantheline) and became very pronounced with higher doses. Methantheline, given intravenously, has been claimed to decrease asthmatic obstruction in man (Sjoerdsma and Dodge, 1954). Buscopan has had a similar effect in a few cases of chronic asthma in which I have tried it. According to some authors khellin is beneficial in bronchial asthma, but this has not been confirmed by others. Pethidine has, in addition to its sedative effect, a spasmolytic action, and it is sometimes used as an anti-asthmatic drug. In the present experiments it had, in contrast to khellin, a definite anti-anaphylactic action, although this became pronounced only with rather high doses.

Salicylic acid and phenylbutazone were investigated because of the frequent experience that salicylates, for instance aspirin, can cause a temporary improvement in an asthmatic attack. The lack of any anti-anaphylactic effect contrasts with this clinical experience. Most of the various substances listed in group (c) were also ineffective. Barium chloride (Pfeiffer and Jarisch, 1913) has been claimed to have an anti-anaphylactic effect. Dibenzyline, the only anti-adrenaline agent in this series, was tested because an antihistaminic action has been attributed to it (Kind, 1954). Phenylephrine was included because its anti-anaphylactic effect does not seem to have been investigated before. Heparin (Williams and van de Carr, 1927) and the vitamins ascorbic acid (Hoffmann, 1942) and cyanocobalamin (Traina, 1950; Ogasawa, Asta and Hisada, 1953) did not show any effect. It is possible that these substances, which play an important part in the normal metabolism of some organs, would exert an influence on anaphylactic shock if they were given in repeated doses over a longer period. Caffeine has been used as an anti-asthmatic drug for a long time. Its anti-anaphylactic action is doubtful, as only the very large amount of 100 mg./kg. had a significant effect.

The only substance in group (c) with a marked anti-anaphylactic effect is sodium cyanate (Schütz, 1949). Hawkins (1953) has shown that it has a relaxant effect on the isolated tracheal ring of the guinea-pig (Fig. 1). There is, at present, no explanation for its action. Its application in human therapy appears hardly advisable because of its toxic effects.

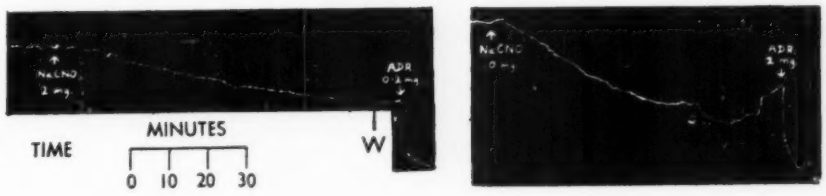


FIG. 1.—Action of sodium cyanate (2 mg. and 10 mg.) on isolated tracheal chains of the guinea-pig, followed by adrenaline (0.2 mg. and 2 mg.) after washing. Bath volume 50 ml. At W, sodium cyanate replaced by fresh Ringer solution. (Hawkins, 1953, unpublished tracing.)

SUMMARY

The anti-anaphylactic effect of nineteen substances has been investigated by the microshock method. Most of them were ineffective, but methantheline, propantheline, buscopan, and sodium cyanate had a strong anti-anaphylactic action.

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# A POSSIBLE MECHANISM OF ADRENALINE STABILIZATION IN THE ADRENAL GLAND

BY

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It has been amply demonstrated that acetylcholine, before its release from nerve endings, exists in the form of a pharmacologically inactive, non-dialysable and denaturable protein complex (Loewi, Hagen, Kohn, and Singer, 1937; Kahane and Levy, 1937; Mann, Tennebaum, and Quastel, 1938, 1939; Brown, 1937). It also seems likely that "sympathin," before liberation from adrenergic nerve endings, must exist there in some stable stored form (Rosenblueth, 1937). In the isolation of adrenaline from the adrenal medulla, Kendall (1932)—in a note not subsequently expanded—reported the occurrence in the gland of lactyladrenaline, stable to hydrolysis, which he suggested might be an intermediate in the formation of adrenaline. The presence of lactyladrenaline and lactylnoradrenaline in hydrolysates of adrenal medullary tumour tissue has been well demonstrated by the careful investigations of Crawford (1951). The compounds were characterized by migration on paper and were compared with synthetic lactyladrenaline and lactylnoradrenaline. He was able to show that these compounds did not exist to any detectable extent in glands which had not been treated with strong acid during the preparation of the extracts, and concluded that they were artifarcts formed in the extraction procedure. Szent-Györgi and Svirbely (1932) also reported that extracts of the adrenal medulla contained a substance which they suggested was an ester of adrenaline.

Utevsky and Osinskaya (1938) and Utevsky and Levantzeva (1938) have suggested that adrenaline in the adrenal gland may be combined with protein. Evidence will be presented in this paper which, it is believed, lends support to this hypothesis in that there appears to be a protein material present in the adrenal medulla which is capable of stabilizing adrenaline and dihydroxyphenylalanine against atmospheric oxidation.

## METHODS

Adrenal glands were obtained from two sources—fresh glands from local abattoirs, and frozen glands donated by the Armour Research Laboratories. The frozen material was prepared by dropping fresh adrenal glands on to dry ice. They were subsequently kept in a deep-freeze cabinet at −20° C., and were not allowed to thaw before use.

After dissecting away the cortex of the gland, homogenates were prepared either by the use of a Waring Blendor, or by grinding the medulla at 4° C. with washed quartz sand in a mortar. The ground material was diluted to a final concentration of 100 mg./ml. with either M/40-phosphate buffer or Krebs-Ringer soln., centrifuged at 800 *G* for 20 to 30 min. at 4° C. to remove cell fragments, and stored in a refrigerator until used. This period varied from 0.5 to 1.5 hr.

It had previously been found that the tissue extract described above would greatly retard the autoxidation of added dihydroxyphenylalanine (DOPA) as well as adrenaline in phosphate buffer at *p*H 7.8 (see Table I and Fig. 1). The effect of the "stabilizer" was therefore measured by comparing the time that elapsed before autoxidation began in solutions of DOPA at *p*H 7.8, and the time required for oxidation of the same amount of DOPA to start in the presence of various amounts of the stabilizer-containing mixture at the same *p*H.

In order to prevent the autoxidation of DOPA before the beginning of the measurement period the substance was dissolved in M/40-monobasic potassium phosphate, which poised the *p*H of the solution at about 5.0, and effectively prevented autoxidation. 0.5 ml. of this mixture and either 0.5 ml. of the monobasic phosphate, or of the solution to be tested, suspended in monobasic phosphate, were placed in the main vessel of the Warburg manometer. When measurements of autoxidation were desired, 1.0 ml. of M/2-dibasic sodium phosphate was added from the side arm, bringing the concentration of phosphate buffer to M/4 and a ratio of secondary to primary phosphate of 20:1, which gave a *p*H of about 7.8. The actual *p*H was slightly lower, owing to the action of the carboxyl group on the DOPA. There was no

TABLE I

THE EFFECT OF VARIOUSLY TREATED AQUEOUS EXTRACTS OF THE ADRENAL MEDULLA ON THE AUTOXIDATION OF DOPA AND ADRENALINE AT *p*H 7·8

All main vessels contained 0·5 ml. of 120 mg.% DOPA in M/40-$KH_2PO_4$ at *p*H 5·1. All sidearms contained 1·0 ml. M/2-$Na_2HPO_4$ at *p*H 8·2. Resulting *p*H on mixing, 7·8.

| No. of Expt. | Added to DOPA in Main Vessel | Added from Sidearm at Mixing | Age of Extract (Hr.) | Length of Lag Period (Min.) | Pre-mix $Q_{O_2}$ (Wet Wt.) | Post-mix | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lag Period $Q_{O_2}$ (Wet Wt.) | Autoxidation $Q_{O_2}$ (Wet Wt.) |
| 1 | M/40-$KH_2PO_4$ | M/2-$Na_2HPO_4$ | | 0<br>0 | 0·16<br>0·00 | —<br>— | 1·13<br>1·11 |
| | Centrifuged extract | M/2-$Na_2HPO_4$ | 24 | 60*<br>45*<br>30* | 0·22<br>0·08<br>0·12 | 0·18<br>0·26<br>0·4 | —<br>—<br>— |
| | Uncentrifuged extract | | | 15<br>0<br>0 | 0·12<br>0·1<br>0·13 | —<br>—<br>— | 0·50<br>0·46<br>0·48 |
| 2 | M/40-$KH_2PO_4$ | M/2-$Na_2HPO_4$ | | 0<br>0 | 0·0<br>0·0 | —<br>— | 1·78<br>1·71 |
| | Centrifuged extract | M/2-$Na_2HPO_4$ | 96 | 15<br>15 | 0·5<br>0·0 | 0·20<br>0·32 | 1·32<br>1·22 |
| | Dialysed extract | M/2-$Na_2HPO_4$ | 96 | 0<br>0 | 0·0<br>0·0 | —<br>— | 1·39<br>1·31 |
| 3 | M/40-$KH_2PO_4$ | M/2-$Na_2HPO_4$ | | 0<br>0 | 0·12<br>0·14 | —<br>— | 1·61<br>1·84 |
| | M/40-$KH_2PO_4$ | Copper $1 \times 10^{-3}$M | 24 | 0<br>0 | 0·10<br>0·08 | —<br>— | 1·74<br>2·04 |
| | Centrifuged extract | M/2-$Na_2HPO_4$ | 24 | 30<br>30 | 0·14<br>0·10 | 0·26<br>0·32 | 2·01<br>2·40 |
| | Centrifuged extract | Copper $1 \times 10^{-3}$M | 24 | 0<br>0 | 0·1<br>0·08 | —<br>— | 2·42<br>2·91 |
| | Uncentrifuged extract | M/2-$Na_2HPO_4$ | 24 | 75*<br>30 | 0·20<br>0·09 | 0·50<br>0·5 | —<br>1·31 |
| | Uncentrifuged extract | Copper $1 \times 10^{-3}$M | 24 | 0<br>0 | 0·14<br>0·13 | —<br>— | 1·66<br>2·31 |
| | Dialysed extract | M/2-$Na_2HPO_4$ | 24 | 75<br>45 | 0·12<br>0·09 | 0·12<br>0·05 | 0·32<br>0·45 |
| 4 | M/40-$KH_2PO_4$ | M/2-$Na_2HPO_4$ | 24 | 0<br>0 | 0·0<br>0·0 | —<br>— | 1·47<br>1·45 |
| | Centrifuged extract | M/2-$Na_2HPO_4$ | 24 | 30<br>30 | 0·04<br>0·1 | 0·28<br>0·30 | 1·62<br>1·42 |
| | Uncentrifuged extract | M/2-$Na_2HPO_4$ | 24 | 45<br>60 | 0·1<br>0·09 | 0·32<br>0·35 | 0·92<br>1·68 |
| | Uncentrifuged extract | $CuSO_4$ $1 \times 10^{-3}$ | 24 | 0 | 0·5 | — | 2·54 |
| | Dialysed extract | M/2-$Na_2HPO_4$ | 24 | 60*<br>90* | 0·0<br>0·1 | 0·19<br>0·24 | —<br>— |
| | Dialysed extract | $CuSO_4$ $1 \times 10^{-3}$M | 24 | 0<br>0 | 0·19<br>0·24 | —<br>— | 0·83<br>0·76 |
| 5 | Centrifuged extract | M/2-$Na_2HPO_4$ | 2 | 105*<br>75*<br>60*<br>105*<br>75*<br>45 | 0·05<br>0·08<br>0·07<br>0·0<br>0·0<br>0·06 | 0·19<br>0·20<br>0·15<br>0·06<br>0·06<br>0·06 | —<br>—<br>—<br>—<br>—<br>0·22 |
| | Centrifuged extract | $CuSO_4$ $1 \times 10^{-3}$M | | 0<br>0<br>0<br>0<br>0<br>0 | 0·02<br>0·08<br>0·16<br>0·08<br>0·03<br>0·06 | —<br>—<br>—<br>—<br>—<br>— | 1·13<br>1·36<br>1·33<br>1·14<br>1·20<br>1·30 |
| 6 | (Added to adrenaline in main vessel) M/40-$KH_2PO_4$ | M/2-$Na_2HPO_4$ | 1 | 30<br>30 | 0·0<br>0·0 | 0·0<br>0·0 | 0·24<br>0·24 |
| | Centrifuged extract | M/2-$Na_2HPO_4$ | 1 | 180*<br>150* | 0·01<br>0·01 | 0·02<br>0·02 | —<br>— |

* Experiment terminated.

measurable difference between the *p*H of the DOPA solution alone and that of the DOPA homogenate when both were treated in this way. Accordingly, any variations in the rate of DOPA autoxidation are not felt to be due to differences in *p*H.

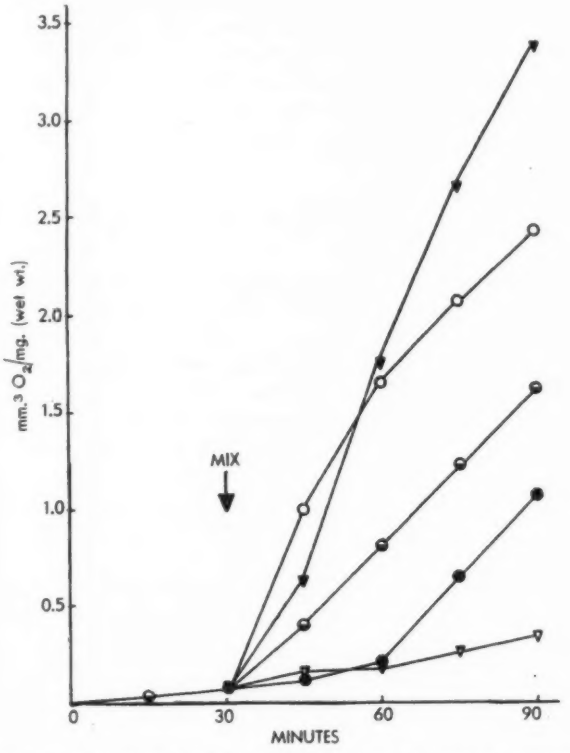


FIG. 1.—Oxidation of DOPA in the presence of variously treated extracts showing the presence of the tissue inhibitor. ●——● DOPA autoxidation (*p*H 7.8). ▼——▼ Boiled extract of medulla. ○——○ Extract of medulla plus $CuSO_4$ ($1 \times 10^{-3}$M). ●——● Extract of medulla. ▽——▽ Dialysed extract of medulla.

It should be emphasized that, under the conditions described above, the result of the addition of secondary phosphate to DOPA in primary phosphate is an immediate uptake of oxygen, and, in a very short time, a darkening of the solution by oxidation products. Thus in the test vessels, if no substance in the extract is capable of stabilizing DOPA against oxidation, the oxygen consumption of the test parallels that of the control. However, in an experiment so arranged, the presence of a stabilizing substance can be detected by a lack of oxygen consumption after the addition of secondary phosphate.

This test was chosen to avoid the necessity of depending upon the presence of adrenaline in the extract. Some of the fractions examined were free of adrenaline, and the addition of DOPA ensured the presence of an autoxidizable substance. DOPA was chosen rather than adrenaline because it was available in a pure state. The experiments have also been run with adrenaline and the results are in complete agreement.

## RESULTS

Table I shows the effect of various treatments on the capacity of homogenates of the adrenal medulla to delay the autoxidation of added adrenaline and DOPA. All fractions were prepared in M/40-monophosphate at *p*H 5.0. It can be seen from Table I that the presence of the tissue extract is capable of retarding the autoxidation of DOPA and adrenaline for a considerable time, depending upon the age of the extract and the treatment it has received.

Table I also shows that the amount of oxygen consumed by the boiled extract in the presence of DOPA greatly exceeded the autoxidation of DOPA, and also the oxygen consumed by the unboiled material after the addition of DOPA. It seems likely that the extra oxygen consumption reflected the oxidation of liberated naturally occurring autoxidizable substances in the gland, which presumably were combined in the fresh gland in a stable complex.

The age of the tissue extract appears to have an effect on the activity of the "stabilizer." In Table I, Expts. 1 and 2 were performed on the same tissue extract, except that before Expt. 2 it had been kept for three days in the refrigerator in M/40 monophosphate buffer. When the extract was tested a second time the lag period was significantly reduced. The reduction in "stabilizing" efficiency was actually greater than would appear from the table, since the one-day-old homogenate had permitted no oxidative activity at the time the experiment was ended. The "ageing" effect was accelerated by increasing the temperature at which the homogenate was kept.

Copper, cobalt, and manganese were tested at a concentration of $1 \times 10^{-3}$M in order to determine what effect heavy metals might have on the system under consideration. The addition of copper abolished the lag period completely—as did cobalt and manganese—and increased the oxygen consumption markedly.

Dialysis of the tissue extract against distilled water enhanced the "stabilizing" capacity of the tissue extract. The dialysate, when tested, showed no "stabilizer" activity and, in fact, it autoxidized spontaneously when the *p*H was raised to 7.8. This observation suggested that the augmented action of the dialysed extract was due to the removal of naturally occurring materials from the complex, which made a greater amount of the stabilizer available for combination with the added DOPA.

The effects of boiling, and of dialysis, upon the tissue extract suggested that the retarding of oxi-

dation might be associated with the protein fraction of the extract. Accordingly, three protein precipitants were tested for their effect on the stability of DOPA in the presence of homogenates, as shown in Table II. Table II indicates clearly

TABLE II

DECREASE IN DOPA-STABILIZING CAPACITY OF ADRENAL MEDULLA EXTRACTS AFTER TREATMENT WITH VARIOUS PROTEIN PRECIPITANTS

All main vessels contained 0.5 ml. of 120 mg.% DOPA in M/40-$KH_2PO_4$ at *p*H 5.1, and 0.5 ml. of soln. to be tested in monophosphate buffer. All sidearms contained 1.0 ml. M/2-$Na_2HPO_4$ at *p*H 8.2. *p*H after mixing 7.8.

| Solution Tested | Test | | | Length of Lag Period (Min.) | Pre/Post-mix mm.$^3$ $O_2$/mg./hr. |
|---|---|---|---|---|---|
| | Millon | Biuret | Ninhydrin | | |
| Phosphate buffer .. | | | | 0 | 0/0·9 |
| Extract .. .. | ++ | ++ | ++ | 90* | 0/0 |
| Extract after treatment with: | | | | | |
| 5% trichloroacetic acid .. .. | − | − | − | 30 | 0/0·76 |
| Zinc sulphate } Barium hydroxide } | − | − | − | 30 | 0/0·76 |
| 2% phosphotungstic acid .. .. | ± | ± | ± | 60 | 0/0·13 |

++ = strong positive. ± = weak positive. − = negative.
* Experiment terminated after 90 min.

that the presence of proteins in the extracts correlates completely with the stabilization phenomenon. For example, with 5.0% trichloroacetic acid and the zinc-barium reagents—and where the extracts were negative to the Millon, biuret and ninhydrin tests—the resulting protein-free solution did not delay the autoxidation of DOPA beyond the first reading period. However, with phosphotungstic acid-treated solutions—where the tests for proteins were all weakly positive—some retarding effect on the autoxidation of DOPA and adrenaline was observed. In the untreated extract no autoxidation could be detected even at the end of the experiment, whereas the controls, containing DOPA, but no extract, at the same *p*H, showed no lag period; and autoxidation proceeded immediately after the addition of secondary phosphate. From these results it seems necessary to conclude that there is a correlation between the extent to which proteins are removed from the adrenal homogenate and the rapidity with which DOPA is oxidized in the solution.

Since it had been suggested that some protection against autoxidation of adrenaline might be furnished by various sulphydryl compounds, or by ascorbic acid, determinations were carried out to assess the role of these compounds in the stabilization of the hormone in gland extracts.

Experiments performed in the presence of M/1000-sodium iodoacetate, an effective inhibitor of sulphydryl-group action, showed no decrease in the time elapsed before autoxidation occurred. In the ammonium sulphate precipitates, the nitroprusside test for sulphydryl compounds proved negative. Accordingly, it is felt that compounds of this type cannot account for the observed stabilization.

Analyses, using the 2:4-dinitrophenol method, detected little or no ascorbic acid, either before or after autoxidation proceeded. Therefore, this compound could not have accounted for the observed stabilization.

Furthermore, stronger evidence than the foregoing may be found upon consideration of the results obtained with 6% trichloroacetic acid. This precipitant removes proteins, most proteoses and peptones, and even some amino acids, but leaves behind sugars and ascorbic acid. One would expect, therefore, if ascorbic acid were instrumental in the stabilizing effect, that the trichloroacetic acid fractionation would result in enhanced stabilizer activity in the protein-free fraction. This did not occur; ascorbic acid may accordingly be eliminated as a cause of the adrenaline stabilization reported in this paper.

Fractionation procedures are being carried out in an attempt to show that it is possible to obtain a purified protein, which, when introduced into solutions containing DOPA or adrenaline, will retard the oxidation of these substances. The addition of saturated ammonium sulphate solution, to a final concentration of 55% saturation, resulted in the sedimentation of a large proportion of the "stabilizer" activity. Further purification of this fraction is being carried out.

## DISCUSSION

The remarkable stability of adrenaline against autoxidation in extracts of the adrenal medulla suggests that adrenaline does not exist in the gland in the same chemical state as after chemical extraction, as pointed out by Kendall (1932) and by Rosenblueth (1937).

Having eliminated the possibility that the redox properties of ascorbic acid, and of compounds containing free sulphydryl groups, are responsible for the observed stability, several alternative explanations may account for the differences in chemical properties between the adrenaline in the gland and the isolated product. Kendall (1932) isolated lactyladrenaline from the adrenal medulla. Crawford (1951), however, cast considerable doubt on the possibility that lactyladrenaline represents the actual storage form of the hormone, but sug-

gested that it is formed as a result of the extraction procedure employed.

Another possibility is that adrenaline does not exist in the gland at all, but that the vasopressor substance is enzymatically produced and immediately liberated from the gland, upon appropriate stimulation. This supposition has a logical weakness which, while not fatal, argues against it. Standard methods for the extraction of adrenaline usually begin with a treatment of the minced gland with 95% ethanol, or some other equally efficient protein precipitant. After a short time such treatment would render normal enzymatic changes most unlikely, and one would expect that the extraction procedure would yield large quantities of an inactive precursor, but little or no formed adrenaline. No such precursor has been identified in these extracts, and large amounts of adrenaline have been obtained.

A third possibility, which would account for both the chemical differences between synthetic adrenaline and the glandular vasopressor substance, is that the vasopressor principle is combined with a protein in the gland in such a manner that, upon demand, adrenaline may be released into the solution. Such a proposal would require as a prerequisite a protein capable of combining with adrenaline, and one which would, in this combination, form a substance which was non-diffusible and non-oxidizable under living conditions. This protein would also have to be related to the physiological activity of the gland in such a manner that stimulation of the gland would result in the destruction of the adrenaline-protein complex, and liberation of the hormone into the circulation.

The evidence presented in this paper indicates that there exists in the adrenal gland a protein which can prevent the autoxidation of adrenaline and DOPA. Although our experiments were not designed to localize adrenaline on particulate cellular components, our data are entirely consistent with the observations of Blaschko and Welch (1953), who showed that adrenaline is associated with the protein-rich mitochondrial fraction of the cells of the adrenal medulla.

## Summary

1. The metabolism of the adrenal medulla has been studied on extracts in the presence of various substrates.

2. Protein, capable of preventing the autoxidation of dihydroxyphenylalanine at *p*H 7.8, exists in the adrenal medulla.

3. It is suggested that this protein acts as a "stabilizer" by forming a complex with adrenaline in the gland and so preventing the autoxidation of adrenaline under physiological conditions.

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# A SERIES OF POLYMETHYLENE BIS-ACETOXYETHYL-DIMETHYLAMMONIUM SALTS

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Polymethylene bis-acetoxyethyldimethylammonium salts may be regarded as consisting of two molecules of acetylcholine (ACh) linked together by a polymethylene chain through a methyl group on each nitrogen atom—

$$CH_3COOCH_2CH_2\overset{+}{N}Me_2\text{-}(CH_2)_n\text{-}Me_2\overset{+}{N}CH_2CH_2OOCCH_3, 2X^-.$$

They were prepared because it was thought that a compound in which the polymethylene chain was of suitable length might be able to combine with two sets of receptors (on structures affected by ACh) at once. Replacement of one methyl group, attached to the quaternary nitrogen atom in ACh, by an ethyl group lowers activity to between one-half and one-fifth (Holton and Ing, 1949); it was felt, therefore, that replacement by a polymethylene chain should not completely destroy affinity for the receptors.

Such a compound, which might be called "pharmacologically bivalent," should be much more than twice as strongly adsorbed at the biological surface as ACh for two reasons (cf. Barlow and Ing, 1948). Firstly, when one end of a "pharmacologically bivalent" molecule dissociates, the drug is still held to the biological surface at the other end, and it is therefore very likely that the dissociated end will become re-adsorbed. Secondly, if the sets of receptors are some distance apart, there may be considerable Van der Waals attraction between the polymethylene chain and the biological surface, if these can fit closely together.

Ethylene bis-acetoxyethyldimethylammonium chloride was prepared and tested by Ing, Kordik, and Tudor-Williams (1952) and found to be inactive. This did not deter me from making other members of this series; I considered it unlikely that a compound with so short a chain could combine with two sets of receptors at once.

The compounds examined were tetra, penta, hexa, hepta, octa, nona, deca, undeca, and dodecamethylene bis-acetoxyethyldimethylammonium salts, and penta, octa, nona, deca, undeca, and dodecamethylene bis-hydroxyethyldimethylammonium salts. Members of the first series are called the *acetoxy* compounds, members of the second the *hydroxy* compounds.

## METHODS

The compounds were tested by standard methods on the frog rectus, frog heart, spinal cat, and rat diaphragm preparations, and on the cholinesterases of dog caudate nucleus and horse serum. Solutions of the *acetoxy* compounds were freshly made up for each pharmacological experiment and made acid to *p*H 4. This eliminated complications arising from hydrolysis, which occurred if the solutions were stored for an hour or two.

In the cholinesterase investigations rates of hydrolysis were determined by measuring the volume of carbon dioxide liberated from the buffer solution (Krebs bicarbonate Ringer) during the first 6 min. Blank experiments were made to determine the extent of spontaneous hydrolysis, which was found to be small in the conditions employed (*p*H 7.2; 37° C.).

## RESULTS

*Experiments on Frog Rectus, Frog Heart, and Spinal Cat.*—Only the nona, deca, undeca, and dodecamethylene members of the *acetoxy* series had any activity. The number of molecules of any of the other compounds required to produce an effect comparable with that of one molecule of ACh was greater than 5,000. The results are summarized in Table I. There is a sharp maximum in activity on all three preparations at the decamethylene compound.

*Experiments on the Rat Diaphragm Preparation.*—Members of the *acetoxy* series antagonized the actions of both decamethonium and (+)-tubocurarine chloride on this preparation. The com-

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TABLE I

ACTIVITY OF *ACETOXY* COMPOUNDS ON FROG RECTUS, FROG HEART, AND CAT BLOOD PRESSURE

$CH_3COOCH_2CH_2\overset{+}{N}Me_2\text{-}(CH_2)_n\text{-}Me_2\overset{+}{N}CH_2CH_2OOCCH_3$

| *n* | Equipotent Molar Ratios | | |
|---|---|---|---|
| | Frog Rectus | Frog Heart | Cat Blood Pressure |
| 9 | 4,317±438 | 1,760 | 704 |
| 10 | 13·3±0·5 | 26·3±4·1 | 34·5 |
| 11 | 156±21 | 198±27 | 472 |
| 12 | 142±26 | 158±31 | 463 |

The table shows the number of molecules of the compound (±S.E.) required to produce effects comparable with those of one molecule of ACh. A high number indicates a low activity. Each value for the frog rectus and frog heart is the mean of 3 expts.; those for the cat blood pressure are the results of 1 expt. only.

pounds are, however, only "anticurare" agents; decamethonium has a paralysing action on the rat diaphragm preparation. When given in a dose of 0.5 mg., members of the *acetoxy* series produced between 12 and 59% reduction of the effects of 1 mg. decamethonium on the preparation. There did not appear to be any great variation in activity with the length of the polymethylene chain.

Members of the *hydroxy* series were more active. Approximately the same effect—between 14 and 50% reduction of the effects of 1 mg. decamethonium—was produced by doses as small as 10 μg. of the penta, nona, deca, undeca, and dodecamethylene compounds (those tested). Dr. E. J. Zaimis has kindly examined decamethylene bis-hydroxyethyldimethylammonium in some detail and finds that its "anticurare" properties are suggestive of moderate anticholinesterase activity (see below). The compound did not antagonize the effects of decamethonium on the cat tibialis preparation.

*Experiments on Cholinesterases.*—Members of the *acetoxy* series were tested as substrates of the cholinesterases of dog caudate nucleus and horse serum. The rates of hydrolysis of the compounds ($10^{-3}$M) were compared with that of ACh ($10^{-3}$M); it must be borne in mind that the compounds contain two ester links whereas ACh contains only one. Both the links were found to be hydrolysed by the enzyme preparations and the total amount of carbon dioxide was about twice that liberated when ACh was the substrate. Comparison of the rates of hydrolysis, however, is complicated by the fact that the hydrolysis of the *acetoxy* compounds must proceed via the monoester.

With the preparation from dog caudate nucleus, there was a sharp maximum in the rates of hydrolysis at the octamethylene compound (Table IIA); this was hydrolysed at about 60% of the rate of ACh.

There was no similar sharp maximum in the rate of hydrolysis by horse serum (Table IIB); there was a rise from the tetramethylene to the pentamethylene compound, but the rate of hydrolysis of the higher members was much the same as that of the pentamethylene compound.

Experiments were also performed in which an *acetoxy* compound ($10^{-3}$M) was incubated together with ACh ($10^{-3}$M). With the cholinesterase of dog caudate nucleus the rate of liberation of $CO_2$ from the buffer solution was less than that during an action on ACh alone. When horse serum was used, the rate of liberation of $CO_2$ by mixtures of ACh and the lower members (tetramethylene to octamethylene) was slightly greater than that during an action on ACh alone; from mixtures containing the higher members the rate was less. If the *acetoxy* compound did not affect the hydrolysis of ACh (or vice versa), the amount of $CO_2$ liberated during the first 6 min. should be the sum of that produced by each compound separately during that time. In Table II, column (ii), the volumes of $CO_2$ produced by hydrolysis of the mixtures during the first 6 min. are expressed as

TABLE II

HYDROLYSIS OF *ACETOXY* COMPOUNDS BY CHOLINESTERASES

$CH_3COOCH_2CH_2\overset{+}{N}Me_2\text{-}(CH_2)_n\text{-}Me_2\overset{+}{N}CH_2CH_2OOCCH_3$

| *n* | Rate of Hydrolysis | | |
|---|---|---|---|
| | Of Compound Alone (i) | Of Mixtures: As % of ACh Rate (ii) | Of Mixtures: As % of Sum of ACh Rate and that of Compound (iii) |
| A. By cholinesterase of dog caudate nucleus: | | | |
| 4 | 0 (2) | 92±4 | 92±4 |
| 5 | 4±2 (3) | 92±3 | 91±1 |
| 6 | 8±2 (3) | 80±4 | 72±4 |
| 7 | 30±4 (3) | 70±3 | 51±4 |
| 8 | 61±2 (2) | 58±14 | 29±16 |
| 9 | 36±2 (3) | 39±8 | 28±5 |
| 10 | 29±5 (3) | 31±1 | 23±2 |
| 11 | 12±5 (3) | 19±5 | 18±6 |
| 12 | 9±1 (2) | 12±4 | 9±3 |
| B By cholinesterase of horse serum: | | | |
| 4 | 25±5 (4) | 106±2 | 90±6 |
| 5 | 72±2 (3) | 127±10 | 75±5 |
| 6 | 67±5 (3) | 100±0 | 61±1 |
| 7 | 88±2 (4) | 106±2 | 57±1 |
| 8 | 77±1 (2) | 102±0 | 57±1 |
| 9 | 84±9 (3) | 91±14 | 50±4 |
| 10 | 71±7 (3) | 82±13 | 49±4 |
| 11 | 76±5 (3) | 81±7 | 47±2 |
| 12 | 72±9 (3) | 87±11 | 52±3 |

In these experiments the rates of hydrolysis of ACh ($10^{-3}$M) and of *acetoxy* compound ($10^{-3}$M), alone and together, were measured. Column (i) shows the amount of carbon dioxide liberated during the first 6 min. expressed as a percentage of the amount liberated in the same conditions by ACh. Each value is the mean of a number of experiments (indicated by the figures in parentheses)±S.E. Columns (ii) and (iii) show the amount of $CO_2$ liberated during the first 6 min. by action of the enzyme on a solution containing both the compound and ACh expressed as a percentage of: column (ii), the amount liberated during that time by ACh alone; column (iii), the sum of the amounts liberated during that time by ACh and the compound separately. Each result is the mean of two experiments±S.E.

percentages of the volume produced by hydrolysis of ACh alone in the same time; in column (iii) they are expressed as percentages of the sums of the volumes produced by hydrolysis of ACh and *acetoxy* compounds separately. This last column can be taken as an approximate index of the affinity of the *acetoxy* compounds for the enzyme. A low percentage may indicate that the less rapidly hydrolysed *acetoxy* compound is strongly adsorbed by the enzyme.

Affinity for the enzyme of dog caudate nucleus appears to increase steadily up the series, though the change in increase appears to be greatest between the penta and hexa, hexa and hepta, and hepta and octamethylene compounds. Affinity for the enzyme of horse serum appears to increase only to the hexamethylene compound and to remain steady thereafter.

The *hydroxy* compounds, which are products of the hydrolysis of the *acetoxy* compounds, inhibited the hydrolysis of ACh by the two enzyme preparations (Table III). It appears that the affinity for both enzymes rises with increasing length of the

TABLE III

INHIBITION OF CHOLINESTERASES BY *HYDROXY* COMPOUNDS

$HOCH_2CH_2\overset{+}{N}Me_2\text{-}(CH_2)_n\text{-}Me_2\overset{+}{N}CH_2CH_2OH$

| *n* | 5 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|
| % inhibition: | | | | | | |
| Of dog caudate nucleus esterase | 0 | 78±5 | 80±5 | 89±5 | 100 | 100 |
| Of horse serum esterase .. | 33±1 | 62±2 | 82±6 | 93±1 | 94±2 | 98±2 |

The percentage inhibition is calculated from the amount of $CO_2$ liberated during the first 6 min.; both ACh and the *hydroxy* compounds were present in concentrations of $10^{-3}$M. Each value is the mean of 2 expts. ±S.E.

polymethylene chain. For the cholinesterase of dog caudate nucleus the *p*I 50 of the decamethylene compound was 4.9; for the dodecamethylene compound it was 6.0.

## DISCUSSION

One of the chief difficulties in the assessment of these results is to predict whether increased adsorbability at the biological surface should lead to increased activity. Clark and Raventos (1937) concluded, from experiments with ACh and tetramethylammonium on the rat intestine, frog auricle, and frog rectus preparations, that equiactive doses of the two drugs formed the same number of drug-receptor complexes—i.e., that tetramethylammonium has only one-thousandth of the activity of ACh because one thousand times as many molecules of tetramethylammonium are needed to form the same number of drug-receptor complexes. If the formation of a drug-receptor complex is simply, or strictly analogous to, a physicochemical adsorption, then increased adsorbability should lead to increased activity. The maximum in the pharmacological activity of the members of the *acetoxy* series at the decamethylene compound might, then, be taken to indicate a sharp rise in adsorbability at this chain length. Likewise, the maximum in the rate of hydrolysis by the cholinesterase of dog caudate nucleus at the octamethylene compound might be taken to imply increased adsorbability at the enzyme surface when this chain separates the two quaternary groups. The increased adsorbability might well be caused by the simultaneous attachment of both ends of these molecules.

There are two difficulties in the way of accepting this idea.

Firstly, adsorbability cannot be the only factor governing activity. Compounds closely related to ACh, but differing from it in some important respect, may antagonize it by competing with ACh for the receptors. The more strongly adsorbed the compound, the more powerful an antagonist it should be. It is difficult to know, therefore, whether high adsorbability should be indicated by high agonistic or high antagonistic activity.

Secondly, the ability of the *acetoxy* and *hydroxy* compounds to inhibit the hydrolysis of ACh by the cholinesterase of dog caudate nucleus appears to increase with chain length. However, in the *acetoxy* series there is a fairly sharp rise in this activity up the series to the octamethylene compound—Table II, column (iii)—and it is conceivable that there is some other factor, besides ability to combine with two receptors, which is responsible for further increases in the activity of higher members. The variation with chain length of the ability of polymethylene bis-trimethylammonium salts to inhibit the hydrolysis of acetyl-β-methylcholine by the "true" cholinesterase of laked rabbit red blood cells (Paton and Zaimis, 1949) is remarkably similar; the dodecamethylene compound appears to be the most active. In this series, however, there is no sharp rise to the octamethylene compound, and it is possible that this sharp rise may only occur in a series of compounds which, like the *acetoxy* compounds, contain an ester group and so may be attached to the extremely important "esteratic" site on the enzyme (Wilson and Bergmann, 1950) as well as at the "anionic" site.

These two objections, then, do not rule out the possibility that the receptors in the pharmacologi-

cal preparations studied are so placed that the decamethylene member of the *acetoxy* series can combine with two sets at once, and the receptors in the enzyme of dog caudate nucleus so placed that the octamethylene compound can combine with two sets at once.

If the idea is correct, it might be possible to obtain powerful antagonists of ACh or powerful anticholinesterases by replacing the acetyl groups by suitable "blocking" groups, such as benzilyl, dibutylcarbamyl, or carbamyl. Kimura and Unna (1950) have, in fact, studied such a compound—decamethylene bis-atropinium—but they report that it is only about twice as powerful an antagonist of the muscarine-like effects of ACh as atropine itself (or as methylatropinium, which is about as active as atropine). This might be accounted for by supposing that, perhaps because of the large size of the atropine molecule, the chain length in the pharmacologically bivalent compound must be rather more than ten methylene groups. It is also possible that the presence of the chain on the quaternary nitrogen atom destroys much of the affinity for the receptors—this is certainly true for the quaternary nitrogen atom in ACh—and that the two fragments should be linked at some less important point.

Three things therefore seem desirable. First, the nona, undeca, and dodecamethylene members of the bis-atropinium series should be examined in order to locate the peak in atropine-like activity. Second, a compound such as decamethylene bis-atropinium, which is pharmacologically bivalent, should be compared with the corresponding pharmacologically "univalent" compound, which, in this instance, would be *N*-*n*-amylatropinium and not *N*-methylatropinium. Third, linkage through the quaternary nitrogen atom should be avoided, not only because of the need to preserve the integrity of the quaternary group, but also because, if the sets of receptors are all orientated in the same way, substances such as the *acetoxy* compounds could only combine with two sets transversely and not with two sets in line.

## Chemical Section

Analyses are by Mr. J. M. L. Cameron and Miss M. W. Christie; m.p.s are uncorrected.

The compounds were prepared by one of three methods:

1. Polymethylene dibromide was condensed with an excess of acetoxyethyldimethylamine dissolved in ethanol.

2. Polymethylene dibromide was condensed with an excess of methylaminoethanol dissolved in ethanol. The polymethylene bis-hydroxyethylmethylamine was liberated with alkali, extracted with ether, distilled, and treated with methyl iodide. The polymethylene bis-hydroxyethyldimethylammonium iodide was esterified with acetic anhydride and a few drops of hydriodic acid.

3. Polymethylene di-iodide was condensed with an excess of dimethylaminoethanol dissolved in ethanol. The polymethylene bis-hydroxyethyldimethylammonium iodide was esterified with acetic anhydride and a few drops of hydriodic acid.

The analyses and preparative details are shown in Table IV.

TABLE IV

CHEMICAL DATA

$$ROCH_2CH_2\overset{+}{N}Me_2\text{-}(CH_2)_n\text{-}Me_2\overset{+}{N}CH_2CH_2OR \quad 2X^-$$

| X | R | *n* | Method (See Text) | m.p. | Crystallized From | Found | | | Calculated | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | C | H | N | C | H | N |
| Br | $CH_3CO$ | 4 | (i) | 204–5° (dec.) | EtOH | 40·1 | 7·16 | 5·88 | 40·2 | 7·18 | 5·86 |
| I | H | 5 | (iii) | Dec. above 258° | EtOH/$Et_2O$ | 31·3 | 6·21 | 5·68 | 31·1 | 6·44 | 5·58 |
| I | $CH_3CO$ | 5 | (iii) | 136–7° | EtOH | 35·0 | 6·14 | 4·93 | 34·8 | 6·21 | 4·78 |
| I | H | 6 | (ii)* | 162–3° | EtOH/MeOH | 32·8 | 6·37 | 5·63 | 32·6 | 6·65 | 5·43 |
| I | $CH_3CO$ | 6 | (ii) | 140° | EtOH/EtCOMe | 36·1 | 6·25 | 4·85 | 36·0 | 6·40 | 4·67 |
| I | H | 7 | (ii)* | 115–6° | EtOH | 34·3 | 6·62 | 5·38 | 34·0 | 6·79 | 5·28 |
| I | $CH_3CO$ | 7 | (ii) | 120° | EtOH | 37·1 | 6·37 | 4·87 | 37·1 | 6·57 | 4·56 |
| I | H | 8 | (iii) | 111° | EtOH | 35·6 | 6·71 | 5·39 | 35·3 | 7·06 | 5·15 |
| I | $CH_3CO$ | 8 | (iii) | 150° | EtOH | 38·1 | 6·95 | 4·43 | 38·2 | 6·76 | 4·46 |
| I | H | 9 | (iii) | Sinters 94°, m.p. 124° | EtOH | 36·4 | 7·08 | 5·12 | 36·6 | 7·24 | 5·02 |
| I | $CH_3CO$ | 9 | (iii) | Sinters 91°, m.p. 105° | EtOH | 39·1 | 6·79 | 4·16 | 39·3 | 6·92 | 4·36 |
| I | H | 10 | (ii)* | 104° | EtOH/$Et_2O$ | 37·6 | 7·67 | 5·01 | 37·8 | 7·42 | 4·90 |
| I | $CH_3CO$ | 10 | (ii) | Sinters 114°, m.p. 221° | EtOH/MeCOMe/$Et_2O$ | 40·4 | 7·20 | 4·38 | 40·2 | 7·08 | 4·27 |
| I | H | 11 | (iii) | 147–8° | EtOH/MeCOMe/$Et_2O$ | 38·8 | 7·47 | 4·99 | 38·9 | 7·59 | 4·78 |
| I | $CH_3CO$ | 11 | (iii) | 114° | EtOH/$Et_2O$ | 41·3 | 7·22 | 4·30 | 41·2 | 7·24 | 4·18 |
| I | H | 12 | (iii) | 138–9° | EtOH/$Et_2O$ | 40·2 | 7·50 | 4·69 | 40·0 | 7·75 | 4·67 |
| I | $CH_3CO$ | 12 | (iii) | 127–8° | EtOH/MeCOEt/$Et_2O$ | 41·9 | 7·24 | 3·93 | 42·1 | 7·39 | 4·10 |

* *Hexamethylene bis-hydroxyethylmethylamine*, b.p. 184–190°/2 mm. *Heptamethylene bis-hydroxyethylmethylamine*, b.p. 158–162°/0·6 mm. *Decamethylene bis-hydroxyethylmethylamine*, b.p. 183–7°/0·8 mm.

## Summary

1. A series of polymethylene bis-acetoxy and bis-hydroxy ethyldimethylammonium salts has been prepared and tested on the frog rectus, frog heart, spinal cat, and rat diaphragm preparations, and on the cholinesterases of dog caudate nucleus and horse serum.

2. There was a sharp maximum in activity on the frog rectus, frog heart, and spinal cat preparations at the decamethylene member of the acetoxy series of compounds. Members of the hydroxy series were inactive.

3. There was a maximum in the rate of hydrolysis of the acetoxy series of compounds by the cholinesterase of dog caudate nucleus at the octamethylene member.

4. Members of the hydroxy series slowed the hydrolysis of acetylcholine by the cholinesterase of dog's caudate nucleus ; members of the acetoxy series did so to a lesser extent.

5. These results are not inconsistent with the idea that decamethylene bis-acetoxyethyldimethylammonium can combine with two sets of receptors at once in the frog rectus, frog heart, and spinal cat preparations, and that the octamethylene compound can combine with two receptors at once on the enzyme. This, however, is not the only explanation, and there is evidence that it may be incorrect.


I wish to thank most sincerely Professor J. H. Burn, F.R.S., and Miss Roneen Hobbs, who performed all the pharmacological experiments, Dr. H. Blaschko and Miss Jean M. Himms, who performed all the enzyme experiments, and Mr. N. A. Dobson, who synthesized the octamethylene compounds. I am also grateful to Professor Burn and Dr. Blaschko for their valuable comments on the manuscript. The work was done during the tenure of an I.C.I. Fellowship. Some of the results were communicated to the British Pharmacological Society in July, 1954.

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# THE ANTICHOLINESTERASE ACTIVITY OF POLYMETHYLENE BIS-QUINOLINIUM SALTS

BY

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(RECEIVED NOVEMBER 15, 1954)

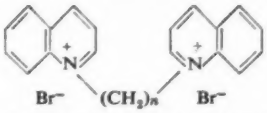
It is known that decamethylene bis-quinolinium (Barlow and Ing, 1948) is a moderately powerful anticholinesterase: Blaschko and Holton (1949), using a preparation of dog caudate nucleus and acetylcholine (ACh) as substrate, recorded a *p*I 50 of 7.0. The hexa, hepta, octa, and nonamethylene members of the series have now been prepared and tested on the enzyme.

## METHODS

The substances were tested for their ability to inhibit the hydrolysis of acetylcholine ($10^{-3}$M) by the cholinesterase of dog caudate nucleus (10 mg. tissue/flask) suspended in Krebs bicarbonate Ringer (*p*H 7.2). The gas phase was 95% $N_2$ and 5% $CO_2$; the temperature was 37°. The volume of $CO_2$ produced during the period from 3 to 9 min. after the start of the experiment was recorded. The amounts produced in flasks containing enzyme, substrate, and inhibitor were compared with those containing enzyme and substrate only. The percentage inhibition of the production of $CO_2$ was plotted against the logarithm of the concentration of inhibitor and the *p*I 50 calculated from the graph. Results (Table I) are the mean of two experiments.

TABLE I

ANTICHOLINESTERASE ACTIVITY OF POLYMETHYLENE BIS-QUINOLINIUM BROMIDES




On preparation from dog's caudate nucleus:

| *n* .. | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|
| *p*I 50 .. | 5·3 | 6·2 | 6·5 | 6·7 | 7·2 |

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## RESULTS

The members of the bis-quinolinium series examined all proved to be inhibitors of the cholinesterase of dog caudate nucleus. There is a steady rise in the *p*I 50 values up the series, without any maximum or even any sudden change from one compound to the next in the series. This absence of a maximum may be considered as support for the idea, previously discussed (Barlow, 1955), that only those bis-onium compounds which are able to become attached to the esteratic sites of the enzyme will show a maximum—or large rise—in affinity at the octamethylene member of the series.

## CHEMICAL SECTION

Analyses are by Mr. J. M. L. Cameron and Miss M. W. Christie; m.p.s are uncorrected.

The compounds were prepared by heating the polymethylene dibromides, dissolved in ethanol, with freshly distilled quinoline in an atmosphere of nitrogen in a sealed tube at 100° for 6 to 12 hours. These precautions were necessary to reduce the formation of bright red products. Some of the compounds crystallized with water of crystallization which was extremely difficult to remove.

*Hexamethylene bis-quinolinium bromide*, recrystallized from ethanol, had m.p. 241–2° (dec.); Found: C, 53.6; H, 5.60; N, 5.36; $Br^-$, 29.8; $C_{24}H_{26}N_2Br_2$, $2H_2O$ requires: C, 53.6; H, 5.63; N, 5.21; $Br^-$, 29.8%.

*Heptamethylene bis-quinolinium bromide*, recrystallized from a mixture of ethanol and ether, had m.p. 150–1° (dec.); Found: C, 56.0; H, 5.62; $Br^-$, 30.3; $C_{25}H_{28}N_2Br_2$, $1H_2O$ requires: C, 56.1; H, 5.67; $Br^-$, 30.0%.

*Octamethylene bis-quinolinium bromide*, recrystallized from a mixture of ethanol and ether, had m.p. 231–2° (dec.); Found: C, 57.1; H, 5.70; N, 5.17;

$Br^-$, 29.0; $C_{36}H_{30}N_2Br_2$, $1H_2O$ requires: C, 56.9; H, 5.90; N, 5.11; $Br^-$, 29.2%.

*Nonamethylene bis-quinolinium bromide*, recrystallized from a mixture of ethanol, methyl ethyl ketone and ether, had m.p. 186° (dec.); Found: C, 57.6; H, 6.00; $Br^-$, 28.6; $C_{27}H_{32}N_2Br_2$, $1H_2O$ requires: C, 57.7; H, 6.11; $Br^-$, 28.5%.

## Summary

The ability of polymethylene bis-quinolinium bromides to inhibit the hydrolysis of acetylcholine by the cholinesterase of dog caudate nucleus increases as the length of the polymethylene chain is increased from 6 to 10 methylene groups.

We wish to thank Dr. H. Blaschko for his interest in this work, which was performed during the tenure by one of us (R. B. B.) of an I.C.I. Fellowship at the University of Glasgow.

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*Brit. J. Pharmacol.* (1955), **10**, 175.

# THE PHARMACOLOGICAL ACTIONS OF CAPSAICIN AND ANALOGUES

BY

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(RECEIVED DECEMBER 8, 1954)

Capsaicin, a pungent principle present in various species of *Capsicum*, is a decylenic acid amide [I,R = $-(CH_2)_4CH:CH.CH(CH_3)_2$] of vanillylamine.

I

On varying the acid portion of the molecule different degrees of pungency of the analogues have been observed (Nelson, 1919 ; Ott and Zimmermann, 1921 ; Jones and Pyman, 1925 ; Ford-Moore and Philips, 1934). Other pharmacological effects of various species of *Capsicum* have been reported. Thus Högyes (1878) first observed that the burning sensation produced by an alcoholic extract of *Capsicum annuum* dropped upon the skin was accompanied by hyperaemia. Lille and Ramirez (1935) found that intravenous injection of an extract of *Capsicum* into the dog produced a fall in blood pressure accompanied by a variable effect on the respiration, an increase in salivary secretion and a relatively small increase in gastric secretion. They also observed that capsaicin extracts increased the activity of the isolated rabbit intestine and guinea-pig uterus, although Nast (1923) had previously found that there was a relaxation of the isolated rabbit intestine. Capsaicin also lowers the rectal temperature of the mouse when given subcutaneously (Issekutz, Lichtneckert, and Nagy, 1950)—an effect interpreted as being due to excitation of heat receptors.

Heubner (1925) found that undecylenacidic—allinyl—amide, an acrid substance related to capsaicin, caused sweating of the forehead of man when smeared upon the mucous membrane of the mouth. In a study of gustatory sweating in healthy people Lee (1954) used the fresh fruits of *Capsicum frutescens, L.*, or *C. minimum, Roxb.* He produced evidence that the reflex was initiated by pain fibres and showed that reflex sweating could not be induced by application of *Capsicum* to the mucosa of the oesophagus or stomach. He found, however, that *Capsicum* produced profuse salivation when placed in the mouth. The oleoresin of *Capsicum* has long been recognized in the *B.P.* and *U.S.P.* as a powerful gastro-intestinal stimulant and as a rubefacient.

The effects obtained with whole fruits or crude extracts of *Capsicum* can no doubt be attributed in part to capsaicin. Since some of the effects described could be due to substances other than capsaicin, the pharmacological actions of pure capsaicin and some of its analogues were investigated on animals with particular reference to the circulation and respiration.

The compounds studied were capsaicin, vanillyl-*n*-decoylamide [I,R = $-(CH_2)_8CH_3$], vanillyl acetamide [I,R = $-CH_3$] and vanillylamine.

## METHODS

Cats under chloralose anaesthesia were used ; the trachea was cannulated and respiration was recorded by measuring the tidal air volume with a closed circuit spirometer filled with oxygen. The blood pressure was recorded either from the femoral or from the carotid artery. Intravenous injections were made direct into the femoral vein, but injections into all other blood vessels were made through cannulae. Injections into the carotid sinus region were made through the lingual artery after clamping the external carotid artery at a point near the origin of the lingual artery, but without tying the occipital and ascending pharyngeal arteries. Injections into the superior mesenteric artery were made through a branch of that artery. Injections into the femoral artery were made through a branch supplying the muscles. Injections into the saphenous artery were made through a cannula inserted into the popliteal artery so that the tip of the cannula was just below the origin of the saphenous artery, and all other branches of the femoral artery were tied off. Injections into the sartorius and quadriceps muscles were made through the femoral artery with the tip of the cannula lying below and pointing towards the origin of the lateral circumflex femoral artery.

Injections into the right auricle were made through a silicone-treated glass catheter which was passed down the left external jugular vein until its tip had entered the auricle. For injection into the left ventricle a similar catheter was passed down the left common carotid artery until its tip had entered the left ventricle. The catheters were filled with a solution of the substance to be injected and 0.2 ml. was injected into the heart each time. At the end of each experiment the chest was opened to determine whether the tips of the catheters were in the chambers of the heart.

Cooling of the vagi was carried out essentially according to the method of Partridge (1939). Dry air, previously chilled by passing through coils covered with frozen $CO_2$, was circulated through hollow tin thermodes on which the vagi lay. By varying the rate of circulation of cold air the vagi could be cooled to the requisite temperature. The temperature of the surface of the thermodes was measured by a thermocouple.

Gastric juice was collected from a catheter inserted into the stomach through an opening in the duodenum, the cat lying on its right side (Wood, 1948).

The vascular effects of capsaicin were studied on the blood vessels of the isolated rabbit's ear which was perfused with Locke solution aerated with oxygen.

Isolated guinea-pig ileum was suspended in 20 ml. Tyrode solution aerated with 95% $O_2$ and 5% $CO_2$. Isolated rat uterus was suspended in 20 ml. of a solution deficient in $Ca^{++}$, prepared according to the formula of Gaddum, Peart, and Vogt (1949) and aerated with $O_2$.

Capsaicin was isolated from the fresh fruits of *Capsicum frutescens, L.*, or *C. minimum, Roxb.* Its isolation and the preparation of its analogues are described in the chemical section at the end of this paper.

Vanillylamine was used as the hydrochloride. Capsaicin and vanillyl-*n*-decoylamide were not readily soluble in water. They were dissolved in 0.1 N-NaOH and the solution was gradually acidified with 0.1 N-HCl until the *p*H was about 7–8. Thus solutions containing 100 μg./ml. capsaicin and 50 μg./ml. vanillyl decoylamide were obtained.

## Results

### *Intravenous Injection*

Injections of 20–40 μg. capsaicin into the femoral vein produced apnoea in the expiratory phase and usually a biphasic effect on the blood pressure, the depressor phase occurring first and being sometimes the only response (Fig. 1). When the vagi were sectioned the apnoeic response and the depressor effect were no longer obtained, but there was a strong pressor effect accompanied by an increase in the respiratory volume. In two out of six cats, however, the depressor effect and decrease in respiratory volume were still obtained but were considerably reduced.

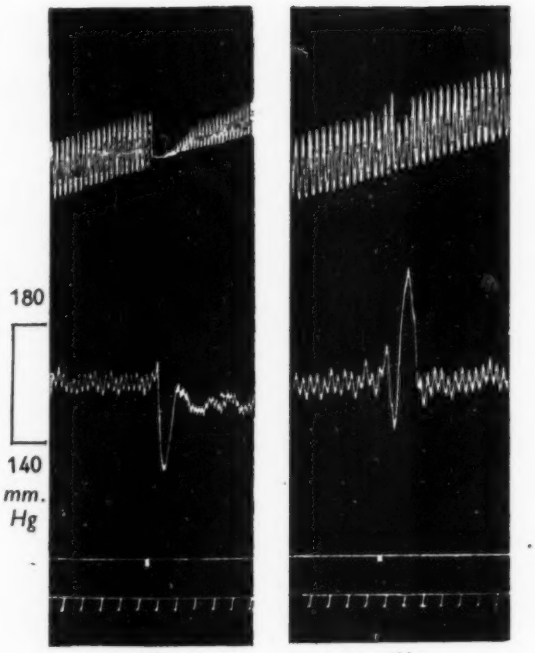


FIG. 1.—Cat, ♂, 3 kg., chloralose. Records: respiration, blood pressure and time (10 sec.). Effect of capsaicin (20 μg.) i.v. Between (*a*) and (*b*) the vagi were cut in the neck.

*Effect of Cooling the Vagi.*—On cooling the vagi with thermodes at 9–10° C. the apnoeic effect of capsaicin was completely abolished in some experiments, but in others it was only reduced. This residual effect on the respiration, however, could be completely abolished by further cooling the vagi to 1.5–3.5° C. (Fig. 2). The depressor effect of capsaicin was more readily abolished by cooling the vagi, the effect disappearing at 8–10° C.

### *Injection into the Heart*

Capsaicin (25 μg.) injected into the right auricle produced apnoea after a latent period of 1.5 sec., but injection into the left ventricle produced hyperpnoea instead. After the vagi were sectioned injection into the right auricle no longer produced apnoea, but injection into the left ventricle continued to produce hyperpnoea (Fig. 3). In the intact animal, a marked depressor effect was obtained almost instantaneously on injection of 25 μg. of capsaicin into either the left ventricle or the right auricle. On sectioning the vagi a pressor effect was obtained in each case.

### *Injection into the Carotid Sinus Region*

When 10 μg. capsaicin was injected into the carotid sinus there was apnoea and a fall of blood pressure (Fig. 4). On sectioning the sinus nerve or the IXth nerve close to its entrance into the

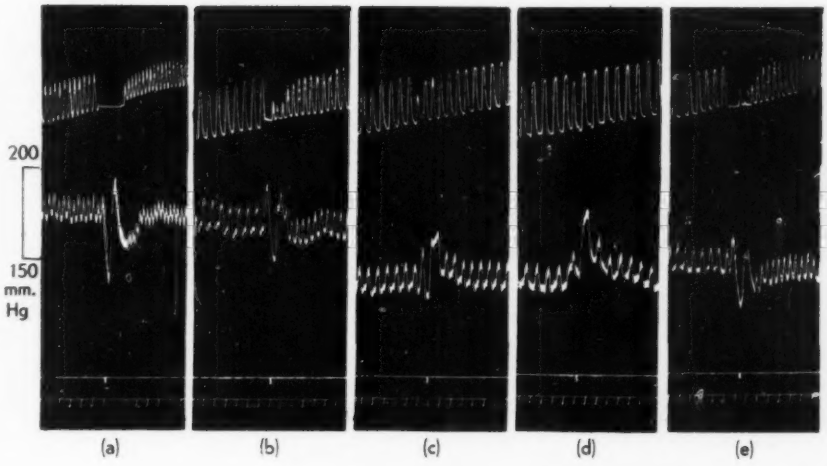


FIG. 2.—Cat, ♂. 3.1 kg., chloralose. Records: respiration, blood pressure and time (10 sec.). The effect of capsaicin (20 μg.) i.v.: (*a*) before cooling cervical vagi, (*b*) on cooling vagi to 8.7° C., (*c*) to 4.1° C., (*d*) to 2.7° C., and (*e*) after recovery from cooling.

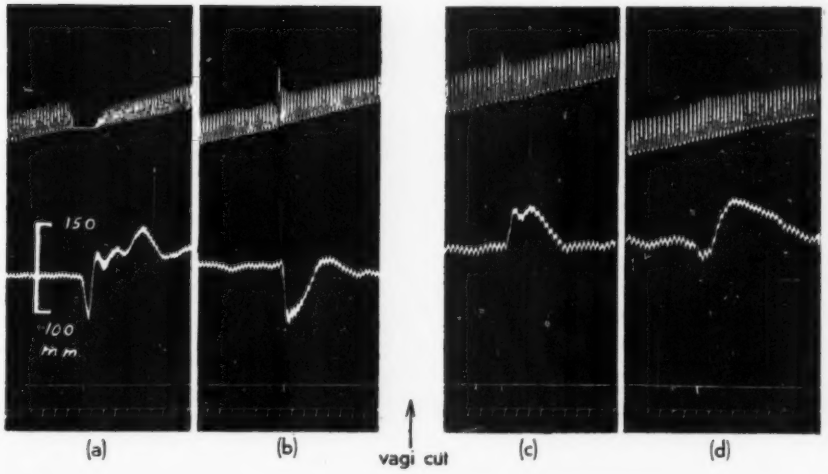


FIG. 3.—Cat, ♀, 3.25 kg., chloralose. Records: respiration, blood pressure and time (10 sec.). Capsaicin (25 μg.) was injected into the right auricle at (*a*) and (*c*) and into the left ventricle at (*b*) and (*d*). Between (*b*) and (*c*) the vagi were sectioned.

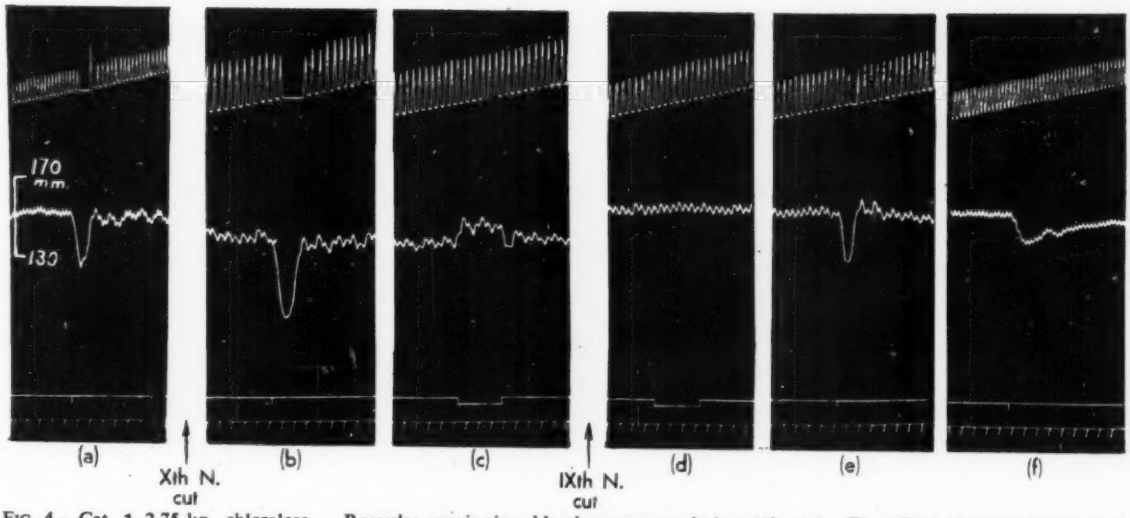


FIG. 4.—Cat, ♂, 2.75 kg., chloralose. Records: respiration, blood pressure and time (10 sec.). The effect of capsaicin (10 μg.) injected into the right carotid sinus at signal marking in (*a*), (*b*), (*e*), and (*f*). The vagi were cut below the sinuses between (*a*) and (*b*). The IXth nerve was cut between (*c*) and (*d*). In (*c*) and (*d*) the right common carotid artery was clamped for the period indicated by the signal marker. In (*f*) the carotid region was covered throughout with a solution of cocaine.

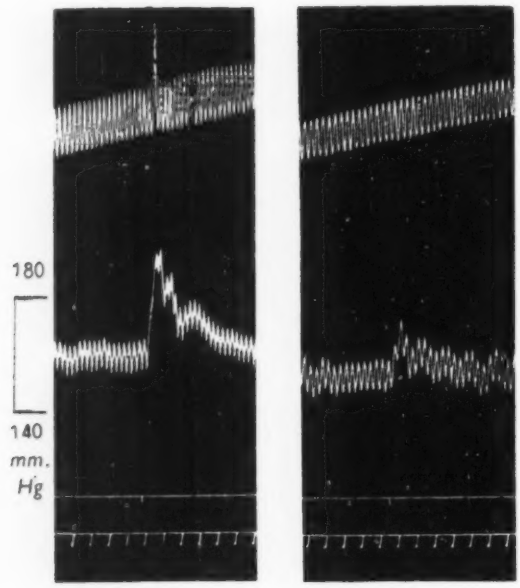


FIG. 5.—Cat, ♀, 2.5 kg., chloralose. Records: respiration, blood pressure and time (10 sec.). Effect of capsaicin (10 μg.) injected into the superior mesenteric artery. The superior and inferior mesenteric nerve plexuses were infiltrated with procaine between (*a*) and (*b*).

skull the respiratory apnoea was considerably reduced in three cats and abolished in one, while the depressor effect was reduced in all the cats but not abolished. When the carotid sinus region and the exposed common carotid artery in the neck were covered with a solution of cocaine, injection of capsaicin into the carotid sinus no longer produced any effect on the respiration, although there was a slow and slight fall in blood pressure which gradually returned to its original level (cf. Fig. 4e and f). This depressor effect, therefore, had its origin elsewhere and it was probably due to capsaicin being carried away through untied vessels, such as the occipital and ascending pharyngeal arteries.

*Injection into the Splanchnic Circulation*

When 10 μg. capsaicin was injected into the superior mesenteric artery, there was a rise in the systemic blood pressure accompanied in two experiments by hyperventilation, and in two others by a decrease in the inspiratory volume (Fig. 5). These responses were not affected by cutting the vagi in the neck, but they were abolished either by extirpating the superior and inferior mesenteric plexuses with their ganglia or by infiltrating these nerve plexuses with procaine. Usually, after abolition of these nervous reflexes, a further injection of capsaicin into the superior mesenteric artery produced a small rise in the systemic blood pressure, although it had no effect on the respiration. This residual effect on the blood pressure is due to mechanical occlusion of the splanchnic vessels brought about by contraction of the intestinal muscular wall in response to capsaicin.

*Injection into the Leg*

When capsaicin (10–20 μg.) was injected into the femoral artery it caused hyperpnoea, sometimes preceded by a brief period of apnoea. The effect on the blood pressure was variable, being either pressor or depressor, and sometimes altogether absent. Twitching of the leg muscles was occasionally observed. These effects were not abolished or reduced on cutting the saphenous and medial cutaneous nerves. In an experiment in which capsaicin (20 μg.) was injected into the saphenous artery pressor and hyperpnoeic effects were observed. These effects were not reduced on skinning the leg, but were completely abolished by sectioning the sciatic and femoral nerves (Fig. 6). These results suggested that capsaicin was acting on nervous receptors in the muscles. On injecting

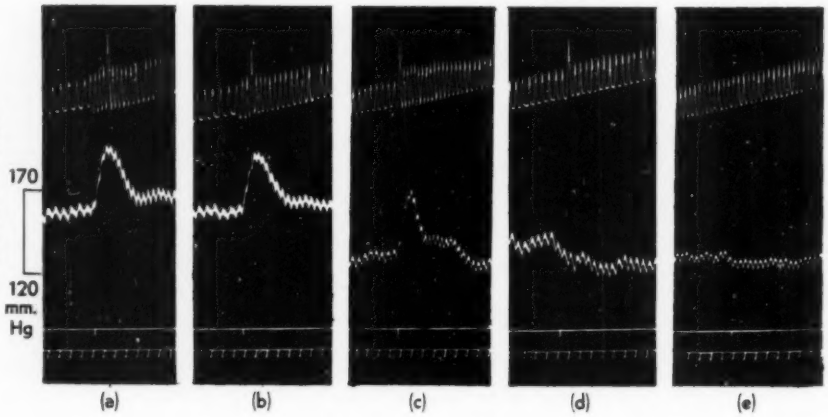


FIG. 6.—Cat, ♂, 3.3 kg., chloralose. Records: respiration, blood pressure and time (10 sec.). Effect of capsaicin (20 μg.) injected into the saphenous artery. The saphenous and medial cutaneous nerves were cut between (*a*) and (*b*). The leg was skinned between (*b*) and (*c*). Between (*c*) and (*d*) the sciatic nerve was cut and between (*d*) and (*e*) the femoral nerve was cut.

capsaicin (10–20 μg.) into the blood supply of the sartorius and quadriceps there was frequently an increased respiratory response accompanied by a slight pressor or depressor effect; all these effects were abolished by sectioning the femoral nerve.

*Other Observations on the Pharmacological Action of Capsaicin*

*Perfused Rabbit Ear.*—Capsaicin in doses as large as 100 μg. did not have any effect on the perfused rabbit ear vessels, although 0.3 μg. adrenaline was sufficient to constrict the blood vessels. In one experiment 10 μg. capsaicin caused a vasodilatation in the ear vessels which could be abolished by a previous injection of procaine (50 μg.) into the perfused ear, but this result could not be repeated.

*Isolated Guinea-pig Intestine.*—Capsaicin contracted the guinea-pig ileum in concentrations of 0.1 μg./ml. The preparation, however, rapidly became tachyphylactic to further doses of capsaicin, although it still responded to histamine or acetylcholine.

*Isolated Rat Uterus.*—Concentrations of 1 μg./ml. capsaicin did not contract or relax the rat uterus, although 0.05 μg./ml. carbachol caused contraction and 0.05 μg./ml. adrenaline complete inhibition.

*Gastric Secretion.*—Capsaicin did not affect HCl secretion in the cat either when injected in doses of 0.5 mg. subcutaneously or when 1 mg. was left in the stomach for 15 min., although a test dose of histamine (0.5 mg.) increased acid secretion when injected subcutaneously. When capsaicin was left in the stomach, there was, however, a small secretion of mucus.

*Pharmacological Activity of Analogues of Capsaicin*

*Vanillylamine.*—Vanillylamine hydrochloride itself did not have any action on the isolated guinea-pig ileum or rat uterus in concentrations of 4 μg./ml. In the cat 5 mg. of the amine injected intravenously had no effect on the respiration, but there was a slight and gradual rise in the blood pressure which occurred about 10–15 sec. after injection quite unlike the sharp rise in blood pressure obtained with capsaicin. The blood pressure gradually returned to its normal value.

*Vanillyl Acetamide.*—This acetyl derivative of vanillylamine, like vanillylamine itself, did not contract or inhibit the isolated rat uterus (50 μg./ml.). The guinea-pig ileum was stimulated by 0.2 μg./ml. vanillyl acetamide, but quickly became tachyphylactic to it. Intravenous injection of 2 mg. into the cat had no effect either on the respiration or on the blood pressure, although a marked apnoea and a strong rise in blood pressure were observed with 20 μg. of vanillyl-*n*-decoylamide.

*Vanillyl-n-decoylamide.*—Vanillyl-*n*-decoylamide produced very much the same effects as capsaicin. However, it had a greater effect on respiration than on the blood pressure, and apnoea could be obtained with small doses of the decoylamide (10 μg.), although a depressor effect on the blood pressure was not always obtained. More often this substance produced a rise in the systemic blood pressure. Vagotomy, or cooling the vagi to 9–10° C., abolished the apnoeic response but not the pressor effect.

The decoylamide produced apnoea when injected in doses of 10 μg. into the right auricle, and this effect was abolished by vagotomy; but injection into the left ventricle increased the depth of respiration and was not affected by vagotomy.

Pupillary dilatation was frequent with intravenous injections of the *n*-decoylamide, even when the superior cervical ganglion was extirpated. The *n*-decoylamide and capsaicin had similar effects on the isolated intestine and uterus.

Capsaicin and vanillyl-*n*-decoylamide produced tachyphylaxis when injected successively at short intervals, but the blood-pressure responses became tachyphylactic more readily than the respiratory responses. Thus, although the effects on the blood pressure became irregular, apnoea could still be readily elicited.

## Discussion

Enough evidence has been collected to show that capsaicin stimulates sensory nerve endings to bring about reflex changes in the systemic blood pressure and respiration which vary with the site of action of the substance. The afferent pathways for these reflex effects include the vagi, the sinus nerves, the splanchnic nerves and the nerves to the skeletal muscles. From the discussion which follows it is concluded that capsaicin, besides acting on chemoreceptors, can also stimulate or sensitize stretch receptors in the lungs and baroreceptors in the carotid sinus.

The apnoeic and depressor effects seen when capsaicin was injected into the chambers of the heart resembled closely those obtained with veratridine (Dawes, 1947; Aviado, Pontius and Schmidt, 1949), 5-hydroxytryptamine (Mott and Paintal, 1953; Schneider and Yonkman, 1953) and the amidines (Dawes and Mott, 1950). The fact that small doses of capsaicin produced apnoea only when injected into the right auricle but not

into the left ventricle suggests that the site of action of capsaicin is probably in the pulmonary circulation. If the observation that cooling of the vagi to about 9–10° C. blocks conduction in slowly adapting pulmonary stretch fibres (Whitteridge, 1948) is related to the present finding that the apnoea produced by capsaicin was greatly reduced or abolished by cooling the vagi to a similar temperature, then it must be concluded that capsaicin acts largely by sensitizing the Hering-Breuer inflation reflex. In some experiments, however, there still remained a small effect on the respiration which was not abolished until the vagi were cooled to a temperature between 1.5° and 3.5° C.; in this respect capsaicin had an action which resembled that of the amidines and which may be mediated by unidentified chemoreceptors in the lungs (Dawes, Mott, and Widdicombe, 1951).

The sudden fall in blood pressure when capsaicin was injected intravenously, or into the left ventricle, is reminiscent of a similar effect first observed with veratrine by von Bezold and Hirt (1867). This effect is partly due to an action on nerve endings in the lungs (Richter and Amann, 1940) and to a greater extent on nerve endings in the heart (Dawes, 1947). Although capsaicin was not injected directly into the coronary arteries, it is very likely that the depressor effect was partly due to a coronary chemoreflex, for it was obtained almost instantaneously with small doses of capsaicin injected into the left ventricle, and it was abolished by vagotomy. Criteria for distinguishing chemoreflexes from the heart and lungs have been given by Dawes and Comroe (1954), but it cannot be ascertained from these whether the depressor effect when capsaicin was injected into the right auricle was a pulmonary depressor effect.

The apnoeic and depressor effects produced on injecting capsaicin into the carotid sinus region were no doubt mediated largely by the sinus nerve. The fact that these effects could not be abolished by sectioning the IXth nerve indicates that other nerve fibres, probably branches from the vagus, also serve as afferent pathways. To what extent the carotid body or the carotid sinus pressor receptors are responsible for these effects has not been determined. It is interesting, however, to suggest the possibility that just as pulmonary stretch receptors can be sensitized so also may baroreceptors in the carotid sinus walls, and it may be the sensitization of these baroreceptors that evoked the reflex fall in blood pressure. Whether the apnoea, if it is at all due to the action of capsaicin on the carotid body, can be related to an increase in blood flow through the carotid body has yet to be determined. An increase in blood flow, however, would appear to be unlikely, as capsaicin has not been found to have a strong vasodilator effect and as there was a fall in blood pressure when capsaicin was injected into the carotid sinus region.

Injection of capsaicin intravenously into an animal whose vagi had been cut at the neck or into the aorta almost always produced a rise in blood pressure and an increase in respiration. These effects are reflex in origin and can be partly attributed to the action of capsaicin in the abdominal viscera and skeletal muscles.

That no reflex effects have been shown to arise from the action of capsaicin on the skin is particularly surprising, as it is well known that it has an irritating action even when spread on the skin. It has not been determined whether the reflex hyperpnoea produced by injecting capsaicin into the muscles was due to stimulation of pain fibres or other nervous receptors. More interesting, however, is the remarkable reflex rise in blood pressure when capsaicin was injected into the splanchnic circulation. Apart from finding that the sympathetic nerve fibres form the afferent pathway, the location of the receptors stimulated has yet to be determined. It would be interesting to find out the physiological role of these receptors in the viscera.

The experiments with the analogues of capsaicin suggest that their pharmacological activity is dependent on the nature of the acyl radical to which vanillylamine is conjugated. Nelson (1919) has shown that there is a relationship between the degree of pungency and the length of the acyl radical. Pharmacological activity may bear a similar relationship to structure: the acetyl derivative of vanillylamine, besides lacking in pungency, had no activity, whereas the acrid decoyl derivatives, with or without an unsaturated bond in the fatty acid chain, showed strong activity.

Capsaicin and its active analogues appear to be potent pharmacological tools for studying the physiological activity of sensory nerve endings.

## Chemical Section

The m.p.s. are uncorrected. Microanalyses were carried out by Dr. W. Zimmermann, C.S.I.R.O., Melbourne.

*Isolation of Capsaicin.*—The method used was a modification of that employed by Tice (1933) and that by Büchi and Hippenmeier (1948). It appears to take a shorter time and may be adapted to the isolation of capsaicin from either the oleoresin or the dried fruit.

Fresh fruit (3 kg.) of *Capsicum frutescens, L.*, or of *C. minimum, Roxb.*, were macerated with acetone (100 g./150 ml. acetone). The acetone was run off the next day and the residue extracted by percolation

3 times more, each time with 2 l. of acetone. The acetone was removed under diminished pressure and the resulting aqueous oily mixture (about 1.8 litres) was shaken with chloroform (500, 300 ml.). The chloroform solution was shaken with 5% NaOH (800 ml.). On the next day the chloroform layer was carefully separated off. Concn. HCl (64 ml.) was added to the alkaline extract to neutralize most of the alkali, and $CO_2$ was passed into the solution until it was no longer blue to thymol blue. The solution was extracted with ether (400, 300, 200 ml.) and after being dried with anhydrous sodium sulphate (20 g.) the ether was removed. The residue was dissolved in 10 times its volume of warm 2% NaOH and any oil remaining undissolved was removed by light petroleum (b.p. 60–80°). Crude capsaicin was precipitated by the addition of a warm saturated solution of ammonium chloride and extracted with ether. Removal of the ether after drying with anhydrous sodium sulphate left an oily residue from which the first crystals were obtained by molecular distillation at 205–215° at 0.3–0.5 mm. In subsequent isolations the oily residue obtained at the last stage was rubbed with light petroleum and seeded with capsaicin, the container being placed in ice. After the oil became semi-solid the mixture was placed in a refrigerator for 24 to 48 hr. The solid mass was filtered off and extracted repeatedly with hot light petroleum (b.p. 80–100°). Crude capsaicin crystallized out from the extracts on chilling and rubbing with a few crystals of capsaicin. The yield of crude capsaicin (m.p. 55–60°) was 0.8–1.5 g. After three recrystallizations from light petroleum the capsaicin melted at 63–64°. (Found : C, 70.3 ; H, 9.0. $C_{18}H_{28}O_3N$ requires C, 70.6 ; H, 9.2%.)

*Other Preparations.*—Vanillylamine and its hydrochloride were prepared according to the method of Challis and Clemo (1947), and vanillyl-*n*-decoylamide according to that of Nelson (1919).

Vanillyl acetamide was prepared by suspending vanillylamine (2 g.) in water (8 ml.) and gradually adding acetic anhydride (2 ml.). After the mixture was heated on a steam bath for 5 min. it was cooled and the amide was extracted with chloroform (3 × 5 ml.). Removal of chloroform after drying gave an oil which crystallized after several days. In subsequent preparations crystals were readily obtained from the oil by rubbing with light petroleum and seeding with crude vanillyl acetamide. Thrice recrystallized material from benzene gave colourless crystals, m.p. 98–99°. (Found : C, 61.90 ; H, 6.85 ; N, 6.9. $C_{10}H_{13}O_3N$ requires C, 61.5 ; H, 6.7 ; N, 7.2%.) Nelson (1919) gave m.p. 84–85°.

## Summary

1. A modified method for isolating capsaicin from *Capsicum* is described.

2. Intravenous injection of capsaicin produces apnoea which is either abolished or greatly reduced by cooling the vagi to 9–10° C. Further cooling the vagi to 2–3° C. abolishes any residual apnoea. This effect is traced to an action on receptors, probably stretch receptors, in the lungs.

3. Capsaicin causes a fall in blood pressure and heart rate which is abolished by cutting the vagi. This effect is probably a coronary chemoreflex.

4. Capsaicin causes apnoea and a fall in blood pressure when it is injected into the carotid sinus region ; these effects are greatly reduced by sectioning the sinus nerve. It is suggested that the depressor effect is due to sensitization of the carotid sinus baroreceptors.

5. Capsaicin acts on undetermined receptors in the intestines, provoking a reflex rise in blood pressure which can be abolished by extirpating the superior and inferior mesenteric plexuses.

6. Capsaicin acts on undetermined receptors in the skeletal musculature, usually causing hyperpnoea and a variable effect on the systemic blood pressure. These effects are abolished by sectioning the nervous supply to the muscles.

7. Capsaicin has no effect on gastric acid secretion.

8. Capsaicin contracts guinea-pig ileum which, however, readily becomes tachyphylactic to the substance. It does not stimulate or inhibit the isolated rat uterus.

9. Capsaicin has no direct action on the blood vessels of the perfused rabbit ear.

10. Vanillylamine and vanillyl acetamide have little or no activity. Vanillyl-*n*-decoylamide has the same pharmacological activity as capsaicin.

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# OBSERVATIONS ON HISTAMINE-INDUCED PRURITUS AND PAIN

BY

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In the course of previous experimental work (Bain, Broadbent, and Warin, 1949) it was noticed that intradermal injections of histamine dissolved in normal saline usually caused pain, but that itching was unusual. These results agreed fairly well with those of Rosenthal and Minard (1939). On the other hand, Melton and Shelley (1950) reported that, although histamine in normal saline did not often produce itching, pruritus was frequent if the saline was buffered. Lewis (1942), however, claimed that itching was predominant even with normal saline as the vehicle. There is, moreover, evidence that itching is common when histamine solutions are applied to the scarified skin (Lewis, 1942; Broadbent, 1953); and, as histamine, present in the skin in an inactive form, may in some circumstances be released to cause itching (Broadbent, 1953), further investigation seemed desirable.

The present paper confirms that the nature of the vehicle in which the histamine is dissolved, and the way in which it is introduced into the skin, are factors in determining the type of skin sensation produced by exogenous histamine.

## METHODS

Histamine acid phosphate was used in all experiments, but the concentrations of histamine are referred to in terms of the base.

Vaccine bottles, containing known quantities of either normal or of buffered saline, were sterilized in an autoclave. The buffered saline had the following composition: NaCl 8.5 g., $NaH_2PO_4$ 0.576 g., $Na_2HPO_4$ 2.5 g., distilled water to one litre.

Histamine solutions used in the experiments were prepared by injecting appropriate volumes of a sterilized stock solution of histamine into the vaccine bottles, with aseptic precautions.

The various solutions used had the following *p*H values, as determined by a glass-electrode *p*H meter accurate to 0.2 of a unit: normal saline, 5.8; buffered saline, 6.8; normal saline with histamine 2 $\mu$g./0.1 ml., 5.3; buffered saline with histamine 2 $\mu$g./0.1 ml., 7.1; buffered saline with histamine 3 $\mu$g./0.1 ml., 7.0. Intradermal injections (0.1 ml.) were made with tuberculin syringes fitted with fine (26 Ga.) hypodermic needles, and scarifications were effected with a sterilized needle. The injections and the scarifications were both performed on the skin of the middle third of the ventral aspects of the forearms. Comparisons were made by applying one solution to the left, and the other to the right, forearm. A short interval, between inserting the needle and injecting the solution, allowed for the subsidence of the pain produced by the introduction of the needle. Scarification was effected by making three parallel scratches through 0.1 ml. of the histamine solution, and then a further three scratches crossing the first series at right angles. The total area of skin affected was about 1 cm.$^2$ With the exception of J. L. B. and B. R. (both of European origin) all subjects were Nigerian medical students. In each experiment the subjects were asked to note the time at which the two solutions were applied to the skin, the type of sensation which ensued—pain or itching—and the time when the sensation ceased. In the experiment, the results of which are detailed in Table I, subjects were, in addition, asked to note which injection was the more painful.

TABLE I

COMPARISON OF THE SENSATIONS PRODUCED BY THE INTRADERMAL INJECTION OF HISTAMINE (2 $\mu$G. IN 0.1 ML.) IN NORMAL SALINE AND IN BUFFERED SALINE

| Subject | Normal Saline | | Buffered Saline | |
|---|---|---|---|---|
| | Duration (min.) of Itch | Pain | Itch | Pain |
| J. J. E. | 0·0 | d.i. | 6·0 | 6·0 |
| I. O. N. N. | 0·0 | d.i.* | 4·0 | d.i. |
| H. N. O. | 0·0 | ,, | 9·0 | ,, |
| F. G. A. M. | 2·0 | ,, | 12·0 | ,, |
| S. M. | 0·0 | 0·5* | 5·0 | ,, |
| U. S. | 7·0 | 4·0* | 15·0 | ,, |
| W. I. B. O. | 0·0 | 6·0* | 12·0 | ,, |
| E. V. J. K. | 0·0 | d.i. | 0·5 | 3·0 |
| C. G. C. | 0·0 | d.i.* | 2·0 | 1·0 |
| E. A. B. | 0·0 | 0·5* | 0·0 | d.i. |
| E. U. A. | 0·0 | d.i.* | 0·5 | ,, |
| B. O. F. | 5·0 | ,, | 0·0 | ,, |
| A. O. O. | 5·0 | ,, | 0·0 | ,, |
| A. E. I. | 1·0 | 1·0 | 8·0 | 2·0 |
| B. R. | 1·0 | d.i.* | 4·5 | 0·0 |
| C. A. I. | 0·0 | d.i. | 2·5 | 1·0 |
| R. B. A. | 0·0 | 2·5* | 10·0 | d.i. |
| Mean | 1·23 | 0·85 | 5·35 | 0·77 |
| S.E. | ±0·52 | | ±1·18 | |

* Denotes that the normal saline injection was more painful than the buffered saline injection.

d.i. = pain during the period of injection only.

## Results

A comparison of the sensations produced by the intradermal injection of 2 μg. histamine in 0.1 ml. normal saline, and in 0.1 ml. buffered saline, respectively, is shown in Table I. It is evident from this Table that histamine dissolved in buffered saline produces a greater incidence and duration of itching ($P<0.05$), but less pain than does histamine dissolved in normal saline. A comparison of the sensations produced by intradermal injection of 0.1 ml. of each of the vehicles alone is shown in Table II. The buffered saline does not cause appreciably more itching than the normal saline.

### Table II

COMPARISON OF THE SKIN SENSATIONS PRODUCED BY THE INTRADERMAL INJECTION OF 0.1 ML. NORMAL SALINE AND OF 0.1 ML. BUFFERED SALINE

| Subject | Normal Saline | | Buffered Saline | |
|---|---|---|---|---|
| | Duration (min.) of Itch | Pain | Itch | Pain |
| R. B. A. .. .. | 0·0 | 0·2 | 0·0 | 2·0 |
| C. A. I. .. .. | 0·1 | d.i. | 0·0 | d.i. |
| L. E. O. .. .. | 0·0 | 0·5 | 0·0 | 1·3 |
| E. A. I. .. .. | 1·0 | 0·0 | 3·0 | 1·0 |
| E. A. M. .. .. | 0·0 | 0·5 | 0·0 | 0·5 |
| B. I. N. .. .. | 0·0 | 0·0 | 0·0 | 2·0 |
| J. L. B. .. .. | 0·0 | 0·1 | 0·0 | 0·0 |
| B. R. .. .. | 0·2 | 0·0 | 0·0 | 0·0 |
| E. F. E. .. .. | 0·0 | 0·0 | 0·0 | 1·0 |
| S. V. E. .. .. | 0·0 | 0·0 | 0·0 | 2·0 |
| E. I. E. .. .. | 1·5 | 0·0 | 1·0 | 0·0 |
| Mean .. .. | 0·25 | 0·12 | 0·36 | 0·89 |

d.i.=pain during the period of injection only.

### Table III

COMPARISON OF THE SENSATIONS PRODUCED BY 3 μG. HISTAMINE IN 0.1 ML. BUFFERED SALINE, WHEN INJECTED INTRADERMALLY AND WHEN APPLIED TO THE SCARIFIED SKIN

| Subject | Intradermal Injection | | Scarification | |
|---|---|---|---|---|
| | Duration (min.) of Itch | Pain | Itch | Pain |
| G. O. S. .. .. | 2·0 | d.i. | 9·5 | 0·0 |
| G. A. .. .. | 2·0 | 0·0 | 10·0 | 0·0 |
| E. F. B. .. .. | 4·0 | 2·0 | 9·0 | 0·0 |
| A. I. .. .. | 6·0 | 1·0 | 22·0 | 0·0 |
| A. O. A. .. .. | 1·0 | 3·0 | 9·0 | 0·0 |
| U. J. E. .. .. | 0·0 | 3·0 | 12·0 | 0·0 |
| N. O. H. O. .. | 0·0 | 3·0 | 19·0 | 0·0 |
| J. L. B. .. .. | 0·0 | 0·5 | 2·0 | 0·0 |
| E. O. I. .. .. | 13·0 | 0·0 | 9·0 | 0·0 |
| E. I. E. .. .. | 0·0 | d.i. | 7·0 | 1·0 |
| E. A. E. .. .. | 6·0 | 2·0 | 9·0 | 0·0 |
| L. E. O. .. .. | 0·0 | 2·0 | 13·0 | 0·0 |
| C. A. I. .. .. | 2·0 | 0·0 | 9·0 | 0·0 |
| R. B. A. .. .. | 8·0 | d.i. | 11·5 | 0·0 |
| Mean .. .. | 3·14 | 1·18 | 10·79 | 0·1 |
| S.E. .. .. | ±1·03 | | ±1·30 | |

d.i.=pain during the period of injection only.

A comparison of the sensations produced by 3 μg. histamine in 0.1 ml. buffered saline when injected intradermally and when applied to the scarified skin is shown in Table III. It is clear that application to the scarified skin causes a higher incidence and a far greater duration of itching ($P<0.001$), but a lesser incidence of pain, than does intradermal injection.

## Discussion

When histamine in normal saline is injected intradermally itching occurs infrequently, but pain is common (Table I). This confirms previous observations (Rosenthal and Minard, 1939; Broadbent, 1953). When histamine is dissolved in buffered saline, both the incidence and duration of itching are increased, thus supporting the observations of Melton and Shelley (1950). As injections of buffered saline alone are not more pruritic than those of normal saline (Table II), the difference may possibly be due to the more painful character of the injections of histamine in normal saline, since pain can inhibit the appearance of itch (Graham, Goodell, and Wolff, 1951). The solutions of histamine in normal saline are more acid than those of histamine in buffered saline.

That histamine in buffer solution is far more pruritic when applied to the scarified skin than when injected intradermally is all the more striking, as nearly all of the histamine solution applied to the scarified skin is seen to stay on the skin surface, and only a minute portion can reach nerve endings, whereas when a similar volume of solution is injected intradermally all the histamine eventually comes into contact with the surrounding tissues. There are several possible explanations of this observation. It may be (1) that dermal oedema interferes with itching (Cormia and Kuykendall, 1953), (2) that pain attending the intradermal injection inhibits the pruritus (Graham, Goodell, and Wolff, 1951), or (3) that the nerve endings subserving the itch sensation lie in the basal layers of the epidermis and are therefore superficial to the injection site (Broadbent, 1953). Such nerve endings would be exposed by scarification of the skin.

It is interesting that the application of histamine to the scarified skin produces lesions resembling those which occur in urticaria in that itching is prominent, whereas itching may be completely absent following the intradermal injection of histamine even when buffered saline is used as the vehicle. Itching is usually absent, but not always so, in dermographism.

SUMMARY

1. Intradermal injections of histamine dissolved in normal saline commonly cause pain. Itching is infrequent.

2. Intradermal injections of histamine dissolved in a special buffered saline cause a greater incidence and duration of itch than do injections of histamine dissolved in normal saline.

3. Intradermal injections of buffered saline alone are not appreciably more pruritic than those of normal saline.

4. When applied to the scarified skin histamine dissolved in the buffered saline causes a greater incidence, and a far greater duration, of itching (but less pain), than when injected intradermally.

The author records his thanks to the Nigerian medical students who acted as subjects for these experiments, to Mr. B. Reiff, who in addition afforded technical assistance, and also to Professor W. A. Bain for advice and criticism.

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# MODIFICATION BY DRUGS OF THE RESPONSE OF THE RAT'S UTERUS TO ADRENALINE

BY

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When adrenaline is assayed by its inhibitory action on the contractions of the rat's uterus elicited by carbachol, the question of the specificity of the inhibition often arises. This happens particularly when impure extracts of biological material are examined. Thus it would be useful to have a specific antagonist of this reaction which could be employed in a way analogous to the use of mepyramine in assays of histamine. A second problem arises in the assay of very small quantities of adrenaline, namely the unpredictability of the sensitivity of the uterine tissue, so that some specimens have to be discarded owing to their high threshold. In the following paper a number of drugs were tested for their suitability either as antagonists or as sensitizers for the inhibitory action of adrenaline on uterine contraction.

In the search for suitable drugs the fact was borne in mind that drug antagonists may, in very small doses, act as sensitizers to the agonist. Thus Gaddum and Kwiatkowski (1938) confirmed and amplified the evidence that this is true of ephedrine. This observation was extended by Jang (1941) to adrenaline antagonists which were chemically less nearly related to adrenaline than is ephedrine. Jang examined the influence of ergotoxine, yohimbine and piperidyl-benzodioxane on several motor effects of adrenaline and found that one of them, the constriction of the vessels in the rabbit's ear, was enhanced by small doses of either of the three drugs. Inhibitory actions of adrenaline were not investigated.

## METHODS

Uterine horns of non-pregnant rats (body weight 120–250 g.) were suspended in a 2 ml. bath containing an aerated salt solution of low calcium content and were stimulated by carbachol at regular intervals (for details see Gaddum, Peart, and Vogt, 1949; Gaddum and Lembeck, 1949). In some experiments both horns were suspended simultaneously in two baths immersed in the same thermostat and the carbachol contractions were recorded on the same drum. The drug under investigation was added to one bath, and any effects on the sensitivity to adrenaline compared with the responses of the untreated horn. Spontaneous changes in sensitivity to adrenaline could thus be distinguished from those produced by the drug.

Most drugs were dissolved in 0.9% NaCl. Exceptions were the members of the halo-alkylamine group (Table II, Nos. 19–21), of which 0.1% stock solutions in propylene glycol were prepared and diluted with 0.9% NaCl immediately before use; "Dibozane" (Table I, No. 3), jervine, pseudojervine, and veratramine, which were dissolved in 3% acetic acid to make 1% or 0.1% solutions; these also were diluted with saline immediately before use; veratrosine, which was dissolved in 0.9% NaCl to which one drop of 0.1N-HCl was added for each 5 ml. of solution; and β-tetrahydronaphthylamine carbonate, 5-benzyloxygramine and "Medmain" (Table II, No. 26), which were dissolved in HCl before dilution with 0.9% NaCl; they were neutralized with $NaHCO_3$ immediately before use.

Two ways were used of exposing the uterus to the action of the drugs under investigation. One was to add the drug to the reservoir of salt solution which did not contain the carbachol; in this way exposure to the drug was continuous except for the 50 sec. in each two-minute cycle in which the muscle was contracting under the influence of carbachol. The other was to add the drug to the bath itself while the flow of salt solutions from the automatic machine was interrupted and to leave it in for (usually) 10 min. The bath was then washed and the normal cycle of carbachol-containing and carbachol-free salt solutions resumed. This short exposure is particularly suited for compounds the action of which is only slowly reversible.

## RESULTS

Table I shows that, of the 27 drugs examined, 18 had no effect on the inhibition of uterine contractions by adrenaline. The table gives only the maximal concentrations in the bath, or in the reservoir feeding the bath, tolerated by the uterus; further increase in the dose produced irregularities or lack of response to the carbachol stimulus. Thus chlorcyclizine and chlorpromazine could only be employed in relatively low concentrations, as they have a fairly strong anti-acetylcholine action.

TABLE I

DRUGS WHICH DID NOT MODIFY THE INHIBITORY ACTION OF ADRENALINE ON THE RAT'S UTERUS

| No. | Compound | Maximal Concentration (mg./l.) Applied to Tissue | |
|---|---|---|---|
| | | For 10 min. | Permanently |
| 1 | Diethylamino-methyl-benzodioxane HCl (883 F) .. | 50 | 1·0 |
| 2 | Piperidyl-methyl-benzodioxane HCl (933 F) .. .. | 5 | |
| 3 | 1, 4 bis (1, 4-benzodioxane-2-methyl)-piperazine ("Dibozane") .. .. | | 10 |
| 4 | Thymoxyethyldiethylamine HCl (929 F) .. .. | 0·5 | |
| 5 | Phenindamine hydrogen tartrate ("Thephorin") .. | 0·5 | |
| 6 | Chlorprophenpyridamine maleate ("Chlortrimeton") | 50 | 1·0 |
| 7 | Chlorcyclizine HCl ("Histantin") .. .. .. | 0·005 | |
| 8 | Chlorpromazine HCl ("Largactil") .. .. .. | 0·25 | 1·0 |
| 9 | Yohimbine HCl .. .. | 50 (5 min.) | |
| 10 | Tolazoline HCl ("Priscol") | 0·5 | 2·5 |
| 11 | Phentolamine ("Rogitine") | 25 | 0·25 |
| 12 | Jervine } Veratrum | 5 | |
| 13 | Pseudojervine } alkaloids | 0·25 | |
| 14 | Veratrosine } | | 5 |
| 15 | Ephedrine HCl .. .. | 5 | |
| 16 | Iproniazid ("Marsilid") .. | 0·5 | 10 |
| 17 | Eserine sulphate .. .. | | 2 |
| 18 | NaF .. .. .. .. | 100 | 1,000 (30 min.*) |

* After that time responses to carbachol irregular.

TABLE II

DRUGS WHICH MODIFIED THE INHIBITORY ACTION OF ADRENALINE ON THE RAT'S UTERUS

| No. | Compound | Effective Concentrations, Applied to Tissue for 10 min. (mg./l.) | Threshold to | |
|---|---|---|---|---|
| | | | Adrenaline | Isoprenaline |
| 19 | Dibenamine HCl .. | 0·05–0·5 | Lowered | Lowered |
| 20 | *N*-(9 fluorenyl)-*N*-ethyl-*β*-chlorethylamine HCl (SKF 501) .. .. | 0·05 | ,, | ,, |
| 21 | *N*-benzyl-*N*-(*β*-phenoxyisopropyl)-*β*-chlorethylamine HCl (SKF 688A, "Dibenzyline") | 0·037–0·25 | ,, | ,, |
| 22 | Dihydroergotamine methanesulphonate (DHE) .. .. | 0·5–5·0 | Raised | Unchanged |
| 23 | Lysergic acid diethylamide (LSD) .. | 0·01–20·0 | ,, | ,, |
| 24 | *β*-Tetrahydronaphthylamine carbonate (*β*-Tetra) | 0·1–2·0 | Lowered | Lowered |
| 25 | 5-Benzyloxygramine | 0·012–0·37 | ,, | Unchanged or lowered |
| 26 | 2-Methyl-3-ethyl-5-dimethylamino-indole("Medmain") | 5·0* | Raised | Raised |
| 27 | Veratramine | 0·005–0·01 | Temporarily raised | Unchanged |

* Added for 70 sec. in a 2 min. cycle.

Many experiments were done with concentrations lower than those listed, and no effect of the drugs was observed.

Table II contains those drugs which produced some modification of the inhibitory effect of adrenaline on contractions due to carbachol. The group of the halo-alkylamines (Nos. 19–21) sensitized to the action of adrenaline. Application of low concentrations of any of these drugs (e.g., 0.037 mg. dibenzyline/l.) for 10 min. was usually effective; prolonged exposure to the same concentration caused interference with the response to carbachol; if this concentration was reduced to one-half, it was tolerated for a longer period, but produced a lesser degree of sensitization. On theoretical grounds one might have expected an antagonism to the action of adrenaline by larger doses of the same drugs. When, however, concentrations from 0.25 mg./l. onwards were applied, even very briefly, the uterine responses to carbachol were suppressed so that their inhibition by adrenaline could not be investigated.

These halo-alkylamines have proved very useful in solving the practical problem of rendering uteri of low sensitivity to adrenaline suitable for the assay of fractions of a nanogram (Holzbauer and Vogt, 1954). There were only a few occasions when sensitization of the uterus was not obtained by one of these drugs. Of the three compounds tested, dibenzyline appeared to be the most effective, though not enough experiments were carried out with the other two compounds to establish the significance of the difference. Fig. 1 shows a tracing of a 20-fold increase in sensitivity to adrenaline by the addition, to a 2 ml. bath, of 0.05 followed by 0.075 $\mu$g. dibenzyline, each dose left in contact with the tissue for 10 min. The tracing also shows that the sensitization affects isoprenaline and adrenaline equally. Experiments on uteri with varying initial sensitivities suggested that insensitive uteri showed a greater lowering of the threshold than sensitive ones, and that responses to less than 0.05 ng. adrenaline (per 2 ml. bath) were unobtainable; it is of interest that this maximal sensitivity which may be obtained artificially is the same as the highest sensitivity occurring naturally in some specimens.

A second group of drugs found to modify the inhibitory action of adrenaline consists of three substances related to the ergot alkaloids. Two of these, dihydro-ergotamine (DHE) and lysergic acid diethylamide (LSD), reduced the inhibitory effect of adrenaline, LSD being more specific than DHE, in that it diminished the sensitivity to adrenaline to between 1/5 and 1/100 of its previous value without interfering with the carbachol contractions; DHE began to inhibit the response to carbachol when the sensitivity to adrenaline was reduced to about one-half. The action of both

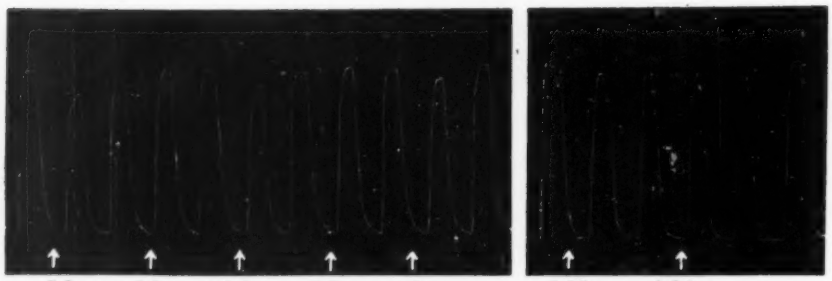


FIG. 1.—Rat uterus. Contractions elicited every 2 min. by carbachol. At the arrows, different doses of adrenaline (A) or isoprenaline (I) added to the 2 ml. bath 45 sec. before the next dose of carbachol is due. Left: sensitivity before dibenzyline. Right: sensitivity after 0.05 μg. dibenzyline added to the bath for 10 min., followed 40 min. later by 0.075 μg. dibenzyline for 10 min. Tracing starts another 24 min. later. Threshold to adrenaline and to isoprenaline lowered approx. 20 times. Doses in nanograms.

substances was specific in the sense that the response to isoprenaline was less affected than that to adrenaline. Fig. 2 is an example of a reduction by LSD of the sensitivity to adrenaline to 1/8 of its original value without any change in the response to isoprenaline. The degree of discrimination between adrenaline and isoprenaline varied from one uterus to the other ; when the discrimination is good, such a uterus is a useful tool in helping to decide whether an inhibition is caused by adrenaline or by isoprenaline, a question which has arisen in the assay of adrenal medullary extracts (Lockett, 1954). The doses required to abolish the responses to adrenaline varied between 0.01 and 20 mg./l., depending on the amount of adrenaline to be antagonized. Neither LSD nor DHE sensitized to the action of adrenaline when applied in lower concentrations (0.005–0.05 mg. DHE/l.; 0.005 mg. LSD/l.).

The third substance from this group, β-tetrahydronaphthylamine ("β-Tetra," No. 24), did not raise but lowered the threshold to adrenaline. The sensitization obtained in a uterus responding initially to 0.25 ng. was about fourfold, and was thus no less than that produced by the haloalkylamines. When the concentration was increased above 2 mg./l., the threshold to adrenaline rose again without ever exceeding the original level. Increase in the concentration of β-Tetra above 40 mg./l. interfered with the effects of carbachol, so that the failure to demonstrate any antagonism to adrenaline may have been due to the limitation in the concentration imposed by the impairment of the response to carbachol. β-Tetra affected adrenaline and isoprenaline to the same extent.

The next two substances tested, 5-benzyloxygramine and medmain, are 5-hydroxytryptamine (HT) analogues, both endowed with an antagonistic action to HT on the rat's uterus (Gaddum, Hameed, Hathway, and Stephens, 1955 ; Shaw and Woolley, 1954). Benzyloxygramine (No. 25) lowered and medmain (No. 26) raised the threshold to adrenaline. Very high concentrations were required to obtain any effect with medmain, and the effects were rapidly reversible. This substance has the peculiarity of being rapidly oxidized in an aerated salt solution (Woolley, personal communication). It was found that the only effective way of using it was to add it to the organ bath as soon as the carbachol was being washed out and to leave it in contact with the tissue for 70 sec., when the next dose of carbachol was due. When this was done repeatedly, the organ was exposed to the drug for 70 sec. in every 2 min. cycle. When tested in this way in the presence of medmain (5 mg./l.), threshold doses of adrenaline and of isoprenaline were antagonized, whereas the response to larger doses was little changed. When more medmain was used, antagonism to the response to carbachol ensued.

In contrast to the antagonistic effect of medmain, 5-benzyloxygramine sensitized to adrenaline, whilst the response to isoprenaline was not consistently

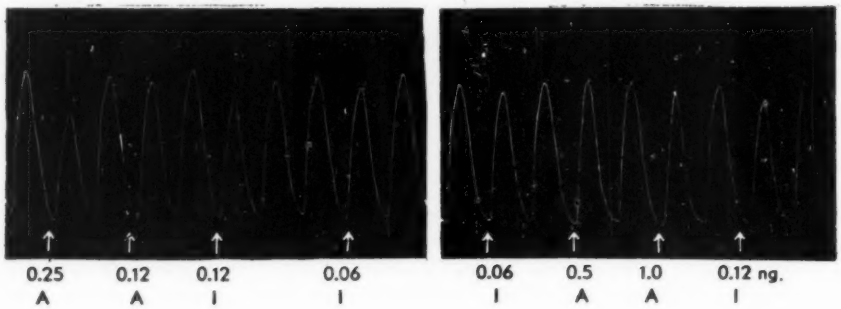


FIG. 2.—Rat uterus. Contractions elicited every 2 min. by carbachol. At the arrows, different doses of adrenaline (A) or isoprenaline (I) added to the 2 ml. bath 45 sec. before the next dose of carbachol is due. Left: sensitivity before LSD. Right: sensitivity after 2.5 μg. LSD added to the bath for 10 min., followed 15 min. later by 5 μg. LSD for 10 min. Tracing starts another 12 min. later. Threshold to adrenaline raised by a factor of 8; threshold to isoprenaline unchanged. Doses in nanograms.

enhanced. It was the only substance encountered which sometimes sensitized to adrenaline without sensitizing to isoprenaline. The effective concentrations varied greatly in different preparations, and so did the maximal concentrations which left the responses to carbachol unimpaired. Inhibition, by higher concentrations, of the response to adrenaline was not observed, possibly because of the limit imposed by the interference of the drug with the stimulation by carbachol. The smallest effective dose of adrenaline after 5-benzyloxygramine was 0.12 ng./2 ml. bath, and thus not much different from that achieved with the halo-alkylamines. For practical purposes the great variability of the antagonism of 5-benzyloxygramine to carbachol would make it a difficult drug to employ in assays.

The last substance found to affect the inhibitory action of adrenaline on the rat's uterus was veratramine. In uteri which had a low threshold to adrenaline, small doses of the drug (0.005–0.01 mg./l.) acted as antagonists and raised the threshold between 2- and 13-fold. The threshold to isoprenaline remained unaltered. The antagonistic effect was, however, evanescent, and disappeared either spontaneously or when larger doses of veratramine were added (0.05–0.5 mg./l.). It was not observed when the initial dose of veratramine was high ; in fact, the larger doses were occasionally seen to sensitize the uterus to the action of adrenaline. However, when the movements of both horns of one uterus were recorded simultaneously and the drug was added to one horn only, so that spontaneous changes of threshold could be taken into account, the sensitization was not obtained consistently.

The drug precipitates slowly when its acid solution is diluted with physiological salt solutions. Therefore, each time a dose was added to the organ bath, it was freshly prepared from the acidified stock solution. It could be seen gradually to precipitate in the bath, and prolonged exposure of the tissue to the drug by adding it to the reservoir containing the salt solution was obviously not possible. It may be that the complex and erratic effects of veratramine on the uterine responses to adrenaline are due to the physical instability of its neutral solutions.

## Discussion

A surprising result of these experiments is the fact that, in spite of the existence of so many adrenaline antagonists, the inhibition exerted by adrenaline on the carbachol contractions of the rat's uterus proved very difficult to abolish. This is only partly due to the fact that inhibitory responses to adrenaline are less easily abolished than motor responses, and mainly to the relative lack of specificity of most adrenaline antagonists, which is shown in this preparation by an interference with the response to carbachol before or simultaneously with an interference with the response to adrenaline.

Of the 27 substances tested, nine were found to have an effect on the inhibition of uterine contractions exerted by adrenaline. It is striking that all nine substances were " specific " antagonists of HT on the rat's uterus in the sense that they inhibited HT in concentrations which had no effect against choline esters. This was known for the halo-alkylamines, for DHE and LSD, and for the two HT analogues Nos. 25 and 26 (Gaddum and Hameed, 1954 ; Gaddum *et al.*, 1955 ; Fingl and Gaddum, 1953 ; Gaddum, 1953 ; Shaw and Woolley, 1954). We found it to be also true of β-Tetra, of which 10 mg./l., and of veratramine, of which doses ranging from 0.01 mg./l. to 0.5 mg./l., raised the threshold to HT, whilst the response to carbachol was hardly changed.

None of the substances which failed to modify the action of adrenaline on the uterus (Table I) is known to antagonize HT specifically. Gaddum and Hameed (1954) failed to demonstrate that either yohimbine or 933 F were specific antagonists of HT, but data for the remaining substances of Table I are unfortunately lacking.

The three halo-alkylamines tested were very similar in their action : a sensitization was obtained with sufficient regularity to be of practical use, provided the doses were kept small so as to avoid interference with the response to carbachol. The limit thus imposed on the dosage made it impossible to decide whether larger doses might have antagonized the action of adrenaline. The position was similar for the other two drugs found to sensitize towards adrenaline, β-Tetra and 5-benzyloxygramine ; the only indication that large doses might have an effect opposite to that of small doses was obtained with β-Tetra, large doses of which abolished the sensitization produced by small doses. The final sensitivity achieved was of the same order for all drugs and never exceeded the highest naturally occurring sensitivity to adrenaline.

The three compounds related to ergot alkaloids were much less uniform in their action than the halo-alkylamines. Two of them (DHE and LSD) were adrenaline antagonists, whereas the third, β-Tetra, sensitized to adrenaline. The antagonism was more clearly seen in LSD than in DHE, probably because LSD, in contrast to DHE, reduced

the response to adrenaline long before affecting the contractions due to carbachol. The effect of both substances was specific in the sense that adrenaline was inhibited at a dosage which left isoprenaline unaffected. Both substances, nevertheless, antagonized HT more readily than adrenaline.

Of the two HT-analogues, 5-benzyloxygramine increased and medmain decreased the sensitivity to adrenaline. The antagonistic effect of medmain was only obtained with large doses and may possibly be of a completely different nature from that of DHE and LSD. An indication that a different phenomenon might be involved lies in the fact that the response to adrenaline and isoprenaline was affected equally by medmain, whereas adrenaline only was inhibited by DHE and LSD.

The action of veratramine, ill-defined as it is, is of some interest in view of the specific antagonism of that substance towards the tachycardia produced by adrenaline (Krayer, 1949).

Amongst the inactive substances, there were such recognized adrenaline antagonists as the benzodioxanes, including the very potent new compound "Dibozane" (Leitch, Liebig, and Haley, 1954), two antihistamines known to be powerful anti-adrenalines (929 F and phenindamine; Bovet and Bovet-Nitti, 1948), and the imidazolines tolazoline and phentolamine. The lack of sensitization obtained by the anti-amine-oxidases ephedrine and iproniazid, the latter being very powerful *in vitro* (Zeller, Barsky, Fouts, Kirchheimer, and Van Orden, 1952) and *in vivo* (Schayer, 1953), shows that the intensity of the response of the isolated uterus to adrenaline is not affected by the activity of amine oxidase in the tissue. Eserine was investigated because Agar (1940) had shown that the inhibitory effect of adrenaline on the guinea-pig's uterus is reversed by pretreating the tissue with eserine. Sodium fluoride was examined on account of the theory put forward by Mohme-Lundholm (1953) that adrenaline acts on the tissues by producing lactic acid, and that NaF, by prohibiting glycolysis, prevents the action of adrenaline. No confirmation of this view was obtained.

A very obvious conclusion from this investigation is the complete unpredictability of the effects which even chemically related drugs exert on the inhibitory effect of adrenaline on uterine muscle.

## Summary

1. Twenty-seven substances were tested for their power to modify the inhibition by adrenaline of the carbachol contractions of the rat's uterus.

2. The halo-alkylamines, and more particularly dibenzyline, sensitize the rat's uterus to adrenaline and isoprenaline and are useful in assays of very small quantities of these compounds.

3. Lysergic acid diethylamide and dihydroergotamine antagonize the inhibitory action of adrenaline on the rat's uterus. Isoprenaline is less affected than adrenaline, so that, in a favourable preparation, this test will distinguish adrenaline from isoprenaline.

4. $\beta$-tetrahydronaphthylamine and 5-benzyloxygramine increased, and medmain decreased, the sensitivity of the rat's uterus to adrenaline; the effects of medmain were too small to be of much practical use. 5-Benzyloxygramine was the only substance encountered which sensitized more consistently to adrenaline than to isoprenaline.

One of us (M. V.) is indebted to the Medical Research Council for a grant towards the expenses of this work. Part of the work was done during the tenure by one of us (M. H.) of a British Council Bursary. Our thanks are due to the following for samples of drugs: to Messrs. Rhône-Poulenc, Paris, for compounds 833 F and 929 F (2214 RP) which we obtained through the courtesy of Dr. R. Wien of Messrs. May and Baker; to Messrs. Smith, Kline and French, Philadelphia, for the three halo-alkylamines; to the Schering Corporation, Bloomfield, New Jersey, for chlorprophenpyridamine maleate; to Glaxo Laboratories, Ltd., for 5-benzyloxygramine; to Dr. O. Krayer, Harvard Medical School, for samples of the veratrum alkaloids; to Dr. Th. J. Haley, Los Angeles, for Dibozane; and to Dr. D. W. Woolley, New York, for medmain.

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# THE SCHISTOSOMICIDAL ACTIVITY OF SYMMETRICAL DIAMINODIPHENOXYALKANES

BY

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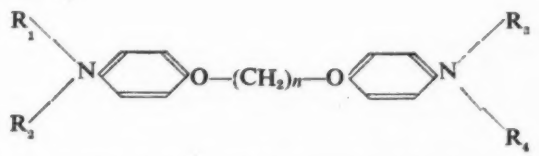
After 1917, when tartar emetic was first used in the treatment of schistosomiasis (Christopherson, 1918), little advance was made in the development of schistosomicides until the lucanthone (miracil) series was synthesized by Mauss (1948). These were the first and only non-antimonial compounds shown to possess significant activity against *Schistosoma mansoni* in the mouse (Kikuth, Gönnert, and Mauss, 1946; Gönnert, 1947; Kikuth and Gönnert, 1948, 1949). They were inactive against *S. japonicum* in experimental animals (Vogel and Minning, 1948).

In November, 1952, we discovered schistosomicidal activity in a chemical type not previously known to possess this property. The compound 1:5-bis(*p*-methylaminophenoxy)pentane dihydrochloride (8C51) was shown to possess marked activity against *S. mansoni* in experimentally infected mice. This discovery stimulated the synthesis of many related compounds with the general formula:




where *n* and R were subject to considerable variation. Of these, nearly 300 were found to possess some activity, many being much more active than the original compound. This paper, the first of a series, describes compounds in which $R_1R_2=R_3R_4$. A preliminary announcement of the discovery of schistosomicidal activity in the series has already been made (Raison and Standen, 1954; Wellcome Foundation, 1954a, b; Burroughs Wellcome & Co., Australia, 1954).

## MATERIALS AND METHODS

Drugs were tested mainly against an Egyptian strain of *S. mansoni* that had been passaged for several years through *Australorbis glabratus*. A Brazilian strain of *S. mansoni* was employed as a check against possible hyper-susceptibility of this long-established strain. *S. japonicum*, passaged through *Oncomelania hupensis*, was used to check against drug-specificity to *S. mansoni*. No tests were made against *S. haematobium*. The white mouse was selected as the most suitable animal for routine tests, but small-scale experiments were also made on guinea-pigs and rabbits.

All animals were infected by the percutaneous route. Each mouse was exposed to 130 cercariae as described by Standen (1949, 1953). Guinea-pigs, under pentobarbitone anaesthesia, were exposed to *S. japonicum* by smearing the shaven belly with an aqueous suspension of 300 cercariae. Rabbits, under pentobarbitone anaesthesia, were infected with *S. mansoni* by exposure to approximately 500 cercariae by insertion of one ear into a cercarial suspension contained in a boiling-tube. Determination of infection in mice was made by identification of viable eggs in the faeces 56 days after exposure to cercariae. Since guinea-pigs do not pass *S. japonicum* eggs in the faeces the presence of infection was established by observation of eggs in mucosa taken at rectal biopsy. In rabbits, which also do not pass eggs in the faeces, the presence of schistosomes was assumed and confirmation made at autopsy.

All drugs were given orally twice daily for 5 days. Any deviation from this standard dosage is specifically indicated, as in single-dose experiments. Drugs were given, in the first instance, at the maximum tolerated dose level or at 200 mg./kg., whichever was the smaller. Where such dosage killed all or some of the schistosomes the experiment was repeated at reduced doses to determine the limits of activity. The animals were killed and examined 7 days after completion of treatment. Schistosomicidal activity was assessed by recording the shift of the worms from the mesenteric veins to the liver, as first described by Schubert (1948) and modified by Standen (1953). In addition it was possible to provide a more precise comparison of activity by noting the proportion of worms *killed* in mice treated with different but related compounds. Whilst the change in worm distribution contributed much to the estimation of the progress of drug action the final assessment of activity was always made on

the proportion of worms killed. Ten mice were used in each test and the activity of a drug was estimated in terms of the total number of worms found in the group. Worms were considered dead only when completely ensheathed with inflammatory tissue and in process of disintegration.

The preparation of the compounds mentioned in this paper followed established chemical procedures (Wellcome Foundation, 1954a, b; Burroughs Wellcome & Co., Australia, 1954).

## Results

### *Routine Examination of Compounds*

The detailed results of the routine examination of compounds for schistosomicidal activity are given in Table I. The term "unit dose" refers to the dose given orally twice daily for 5 days and is therefore one-tenth of the total dose. This table is concerned only with the variation of schistosomicidal activity by symmetrical substitution at both ends of the molecule and by altering the length of the methylene chain; unsymmetrically substituted diaminodiphenoxyalkanes will be dealt with in a further communication. The compounds tested have been grouped according to the variations, listed below, in the general formula shown in Table I.

(i) *Primary amines*: $R_1=R_2=H$ (Table I, A).

(ii) *Secondary amines*: $R_1=H$; $R_2=CH_3$, $C_2H_5$, *n*-$C_3H_7$, *iso*-$C_3H_7$, $C_3H_5$ (allyl), *n*-$C_4H_9$, *iso*-$C_4H_9$, *n*-$C_5H_{11}$ (Table I, B).

(iii) *Tertiary amines*: $R_1=R_2=CH_3$, $C_2H_5$, *n*-$C_3H_7$, $C_3H_5$, *n*-$C_4H_9$; $R_1=CH_3$, $R_2=C_2H_5$; $R_1=CH_3$, $R_2=$*n*-$C_3H_7$, (Table I, C).

(iv) Where necessary the range of values of *n* from 1 to 11 has been covered.

All these compounds have the amino substituents in the *para* positions. Several representatives of the corresponding *meta* and *ortho* series were inactive. The quaternary ammonium ($\dot{N}Me_3$) salts of all three series were also inactive.

### *Comparative Activity of a Diaminodiphenoxyalkane, Lucanthone and Tartar Emetic*

Because of the obviously high activity of the diaminodiphenoxyalkanes, comparative figures were sought for the activity of lucanthone and tartar emetic following single and multiple oral doses. Since many of the diaminodiphenoxyalkanes have a similar order of activity (Table I), the bis-(methylaminophenoxy)heptane (413C52) was selected for these comparative tests. All tests were carried out with mice in groups of 10/dose; most

Table I

STRUCTURES OF DIPHENOXYALKANES AND THEIR SCHISTOSOMICIDAL ACTIVITIES

M.V., mesenteric veins; P.V., portal vein; L., intrahepatic veins. For explanation of "unit dose" see Results.

| Compound No. | *n* | $R_1$ | $R_2$ | Unit Dose (mg./kg.) | Worm Distribution % | | | Worms Killed % |
|---|---|---|---|---|---|---|---|---|
| | | | | | M.V. | P.V. | L. | |
| *A. Primary Amines* | | | | | | | | |
| 76T52 | 2 | H | H | 200 | 69 | 28 | 3 | 2 |
| 333C50 | 3 | H | H | 200 | 0 | 0 | 100 | 100 |
| | | | | 50 | 47 | 17 | 36 | 36 |
| | | | | 25 | 64 | 32 | 4 | 0 |
| | | | | 15 | 65 | 35 | 0 | 0 |
| 334C50 | 4 | H | H | 200 | 0 | 1 | 99 | 99 |
| | | | | 50 | 16 | 5 | 79 | 66 |
| | | | | 25 | 47 | 28 | 25 | 17 |
| | | | | 15 | 74 | 23 | 3 | 0 |
| 373C50 | 5 | H | H | 200 | 0 | 0 | 100 | 100 |
| | | | | 50 | 11 | 0 | 89 | 78 |
| | | | | 25 | 44 | 50 | 6 | 3 |
| | | | | 15 | 73 | 25 | 2 | 0 |
| 335C50 | 6 | H | H | 200 | 0 | 0 | 100 | 100 |
| | | | | 50 | 1 | 0 | 99 | 99 |
| | | | | 25 | 13 | 12 | 75 | 75 |
| | | | | 15 | 76 | 19 | 5 | 3 |
| 153C51 | 7 | H | H | 50 | 0 | 0 | 100 | 100 |
| | | | | 25 | 0 | 3 | 97 | 96 |
| | | | | 15 | 36 | 20 | 44 | 42 |
| 154C51 | 8 | H | H | 200 | 0 | 0 | 100 | 100 |
| | | | | 50 | 0 | 0 | 100 | 100 |
| | | | | 25 | 0 | 6 | 94 | 94 |
| | | | | 15 | 56 | 9 | 35 | 32 |
| 25C53 | 9 | H | H | 50 | 0 | 4 | 96 | 96 |
| | | | | 25 | 15 | 13 | 72 | 72 |
| | | | | 15 | 49 | 19 | 32 | 29 |
| 384C51 | 10 | H | H | 200 | 65 | 27 | 8 | 0 |
| 228C53 | 11 | H | H | 200 | — | — | — | Toxic at this dose |
| | | | | 50 | 78 | 22 | 0 | 0 |
| *B. Secondary Amines* | | | | | | | | |
| 580C54 | 1 | H | $CH_3$ | 200 | 66 | 22 | 12 | 0 |
| 196C54 | 2 | H | $CH_3$ | 200 | 38 | 11 | 51 | 51 |
| | | | | 50 | 70 | 30 | 0 | 0 |
| 410C52 | 3 | H | $CH_3$ | 200 | 0 | 0 | 100 | 100 |
| | | | | 50 | 17 | 21 | 62 | 57 |
| | | | | 25 | 63 | 32 | 5 | 0 |
| 411C52 | 4 | H | $CH_3$ | 200 | 0 | 0 | 100 | 100 |
| | | | | 50 | 0 | 2 | 98 | 98 |
| | | | | 25 | 40 | 15 | 45 | 36 |
| 8C51 | 5 | H | $CH_3$ | 200 | 0 | 1 | 99 | 99 |
| | | | | 50 | 0 | 1 | 99 | 99 |
| | | | | 25 | 31 | 12 | 57 | 55 |
| 412C52 | 6 | H | $CH_3$ | 50 | 0 | 0 | 100 | 100 |
| | | | | 25 | 7 | 3 | 90 | 90 |
| 413C52 | 7 | H | $CH_3$ | 50 | 0 | 0 | 100 | 100 |
| | | | | 25 | 3 | 0 | 97 | 97 |
| | | | | 15 | 46 | 13 | 41 | 41 |
| 414C52 | 8 | H | $CH_3$ | 50 | 0 | 0 | 100 | 100 |
| | | | | 25 | 6 | 3 | 91 | 91 |
| | | | | 15 | 26 | 17 | 57 | 56 |

TABLE I—*continued*

| Compound No. | *n* | $R_1$ | $R_2$ | Unit Dose (mg./kg.) | Worm Distribution % M.V. | P.V. | L. | Worms Killed % |
|---|---|---|---|---|---|---|---|---|
| 26C53 | 9 | H | $CH_3$ | 50<br>25<br>15 | 0<br>10<br>54 | 0<br>13<br>28 | 100<br>77<br>18 | 100<br>77<br>18 |
| 415C52 | 10 | H | $CH_3$ | 200<br>50<br>25 | 40<br>53<br>60 | 19<br>37<br>35 | 41<br>10<br>5 | 39<br>0<br>0 |
| 229C53 | 11 | H | $CH_3$ | 200 | 87 | 13 | 0 | Toxic at this dose |
| 222C54 | 2 | H | $C_2H_5$ | 200<br>50 | 25<br>67 | 8<br>31 | 67<br>2 | 67<br>0 |
| 20T53 | 3 | H | $C_2H_5$ | 200<br>50<br>25 | 0<br>73<br>69 | 0<br>23<br>29 | 100<br>4<br>2 | 100<br>4<br>0 |
| 90C54 | 4 | H | $C_2H_5$ | 50<br>25 | 0<br>18 | 0<br>4 | 100<br>78 | 100<br>78 |
| 201C53 | 5 | H | $C_2H_5$ | 200<br>50<br>25 | 0<br>5<br>50 | 0<br>2<br>42 | 100<br>93<br>8 | 100<br>86<br>0 |
| 115C53 | 6 | H | $C_2H_5$ | 50<br>25 | 0<br>34 | 0<br>28 | 100<br>38 | 100<br>38 |
| 140C53 | 7 | H | $C_2H_5$ | 50<br>25<br>15 | 0<br>1<br>40 | 0<br>3<br>33 | 100<br>96<br>27 | 100<br>96<br>26 |
| 124C53 | 8 | H | $C_2H_5$ | 50<br>25<br>15 | 0<br>7<br>2 | 1<br>3<br>0 | 99<br>90<br>98 | 99<br>90<br>98 |
| 215C53 | 9 | H | $C_2H_5$ | 50<br>25 | 3<br>46 | 2<br>13 | 95<br>41 | 95<br>39 |
| 223C54 | 10 | H | $C_2H_5$ | 200<br>50 | 2<br>73 | 4<br>26 | 94<br>1 | 94<br>0 |
| 411C54 | 11 | H | $C_2H_5$ | 200<br><br>50 | —<br><br>58 | —<br><br>32 | —<br><br>10 | Toxic at this dose<br>0 |
| 234C54 | 2 | H | $n$-$C_3H_7$ | 200<br>50 | 42<br>66 | 49<br>31 | 9<br>3 | 0<br>0 |
| 235C54 | 3 | H | $n$-$C_3H_7$ | 200<br>50 | 5<br>69 | 0<br>25 | 95<br>6 | 95<br>0 |
| 91C54 | 4 | H | $n$-$C_3H_7$ | 200<br>50<br>25 | 0<br>17<br>48 | 0<br>4<br>51 | 100<br>79<br>1 | 100<br>79<br>0 |
| 263C53 | 5 | H | $n$-$C_3H_7$ | 200<br>50 | 0<br>70 | 0<br>11 | 100<br>19 | 100<br>15 |
| 164C53 | 6 | H | $n$-$C_3H_7$ | 200<br>50 | 25<br>61 | 9<br>20 | 66<br>19 | 66<br>16 |
| 165C53 | 7 | H | $n$-$C_3H_7$ | 50<br>25 | 1<br>69 | 2<br>22 | 97<br>9 | 97<br>7 |
| 166C53 | 8 | H | $n$-$C_3H_7$ | 200<br>50<br>25 | 4<br>7<br>63 | 0<br>7<br>29 | 96<br>86<br>8 | 96<br>86<br>0 |
| 271C53 | 9 | H | $n$-$C_3H_7$ | 200<br>50 | 0<br>29 | 0<br>29 | 100<br>42 | 100<br>35 |
| 266C54 | 10 | H | $n$-$C_3H_7$ | 200<br>50 | 65<br>56 | 31<br>39 | 4<br>5 | 0<br>0 |
| 546C54 | 3 | H | *iso*-$C_3H_7$ | 200 | 70 | 28 | 2 | 0 |
| 269C54 | 4 | H | *iso*-$C_3H_7$ | 200<br>50 | 12<br>70 | 0<br>26 | 88<br>4 | 88<br>1 |
| 282C54 | 5 | H | *iso*-$C_3H_7$ | 200<br>50 | 0<br>75 | 1<br>23 | 99<br>2 | 99<br>0 |

TABLE I—*continued*

| Compound No. | *n* | $R_1$ | $R_2$ | Unit Dose (mg./kg.) | Worm Distribution % M.V. | P.V. | L. | Worms Killed % |
|---|---|---|---|---|---|---|---|---|
| 183C53 | 6 | H | *iso*-$C_3H_7$ | 200<br>50 | 43<br>72 | 51<br>27 | 6<br>1 | 1<br>0 |
| 221C53 | 7 | H | *iso*-$C_3H_7$ | 200<br>50 | 53<br>77 | 30<br>22 | 17<br>1 | 17<br>0 |
| 194C53 | 8 | H | *iso*-$C_3H_7$ | 200<br>50 | 56<br>68 | 33<br>30 | 11<br>2 | 11<br>0 |
| 288C54 | 4 | H | $C_5H_5$ | 200<br>50 | 0<br>51 | 0<br>22 | 100<br>27 | 100<br>27 |
| 3C54 | 6 | H | $C_5H_5$ | 50 | 25 | 32 | 43 | 43 |
| 305C53 | 8 | H | $C_5H_5$ | 100<br>50<br>25 | 0<br>6<br>28 | 0<br>8<br>19 | 100<br>86<br>53 | 100<br>86<br>53 |
| 593C54 | 2 | H | $n$-$C_4H_9$ | 200 | 56 | 40 | 4 | 0 |
| 579C54 | 3 | H | $n$-$C_4H_9$ | 200 | 53 | 34 | 13 | 10 |
| 268C54 | 4 | H | $n$-$C_4H_9$ | 200<br>50 | 11<br>66 | 8<br>26 | 81<br>8 | 81<br>0 |
| 296C54 | 5 | H | $n$-$C_4H_9$ | 200<br>50 | 18<br>71 | 18<br>21 | 64<br>8 | 58<br>0 |
| 177C53 | 6 | H | $n$-$C_4H_9$ | 200<br>50 | 74<br>65 | 26<br>20 | 0<br>15 | 0<br>0 |
| 178C53 | 7 | H | $n$-$C_4H_9$ | 200<br>50 | 43<br>64 | 31<br>33 | 26<br>3 | 26<br>0 |
| 179C53 | 8 | H | $n$-$C_4H_9$ | 200<br>50 | 59<br>67 | 30<br>33 | 11<br>0 | 3<br>0 |
| 592C54 | 3 | H | *iso*-$C_4H_9$ | 200 | 79 | 14 | 7 | 6 |
| 567C54 | 4 | H | *iso*-$C_4H_9$ | 200 | 49 | 14 | 37 | 37 |
| 568C54 | 5 | H | *iso*-$C_4H_9$ | 200 | 68 | 21 | 11 | 11 |
| 327C53 | 6 | H | *iso*-$C_4H_9$ | 200 | 45 | 44 | 11 | 0 |
| 328C53 | 7 | H | *iso*-$C_4H_9$ | 200 | 39 | 12 | 49 | 49 |
| 329C53 | 8 | H | *iso*-$C_4H_9$ | 200 | 55 | 39 | 6 | 0 |
| 591C54 | 4 | H | $n$-$C_5H_{11}$ | 200 | 57 | 29 | 14 | 12 |
| 273C53 | 6 | H | $n$-$C_5H_{11}$ | 200<br>50 | 65<br>63 | 32<br>35 | 3<br>2 | 0<br>0 |
| 274C53 | 7 | H | $n$-$C_5H_{11}$ | 200<br>50 | 62<br>78 | 37<br>19 | 1<br>3 | 0<br>0 |
| 275C53 | 8 | H | $n$-$C_5H_{11}$ | 200<br>50 | 58<br>55 | 32<br>43 | 10<br>2 | 0<br>0 |
| *C. Tertiary Amines* | | | | | | | | |
| 195C54 | 2 | $CH_3$ | $CH_3$ | 200 | 47 | 35 | 18 | 15 |
| 416C52 | 3 | $CH_3$ | $CH_3$ | 200<br>50<br>25 | 0<br>54<br>70 | 1<br>39<br>23 | 99<br>7<br>7 | 99<br>0<br>0 |
| 417C52 | 4 | $CH_3$ | $CH_3$ | 200<br>50<br>25 | 8<br>25<br>40 | 7<br>15<br>28 | 85<br>60<br>32 | 85<br>60<br>32 |
| 70C51 | 5 | $CH_3$ | $CH_3$ | 200<br>50<br>25 | 0<br>2<br>27 | 0<br>2<br>27 | 100<br>96<br>46 | 100<br>96<br>46 |
| 418C52 | 6 | $CH_3$ | $CH_3$ | 200<br>50<br>25 | 7<br>19<br>32 | 9<br>17<br>26 | 84<br>64<br>42 | 84<br>64<br>42 |
| 27C53 | 7 | $CH_3$ | $CH_3$ | 50<br>25<br>15 | 0<br>7<br>62 | 0<br>0<br>25 | 100<br>93<br>13 | 100<br>93<br>10 |

TABLE I—*continued*

| Compound No. | *n* | $R_1$ | $R_2$ | Unit Dose (mg./kg.) | Worm Distribution % M.V. | P.V. | L. | Worms Killed % |
|---|---|---|---|---|---|---|---|---|
| 419C52 | 8 | $CH_3$ | $CH_3$ | 200<br>50<br>25 | 28<br>48<br>52 | 25<br>16<br>31 | 47<br>36<br>17 | 46<br>31<br>17 |
| 50C53 | 9 | $CH_3$ | $CH_3$ | 200<br>50<br>25 | 0<br>0<br>44 | 0<br>8<br>36 | 100<br>92<br>20 | 100<br>92<br>20 |
| 420C52 | 10 | $CH_3$ | $CH_3$ | 200<br>50<br>25 | 47<br>76<br>72 | 34<br>22<br>23 | 19<br>2<br>5 | 0<br>0<br>0 |
| 114C54 | 11 | $CH_3$ | $CH_3$ | 200 | 56 | 38 | 6 | Toxic at this dose |
| 401C54 | 2 | $C_2H_5$ | $C_2H_5$ | 200<br>50 | 43<br>62 | 22<br>36 | 35<br>2 | 35<br>0 |
| 116C54 | 3 | $C_2H_5$ | $C_2H_5$ | 200<br>50 | 0<br>59 | 0<br>38 | 100<br>3 | 100<br>0 |
| 407C53 | 4 | $C_2H_5$ | $C_2H_5$ | 200<br>50 | 0<br>0 | 0<br>0 | 100<br>100 | 100<br>100 |
| 162C53 | 5 | $C_2H_5$ | $C_2H_5$ | 200<br>50 | 0<br>18 | 2<br>16 | 98<br>66 | 98<br>66 |
| 82C53 | 6 | $C_2H_5$ | $C_2H_5$ | 200<br>50 | 0<br>22 | 0<br>11 | 100<br>67 | 100<br>62 |
| 296C53 | 7 | $C_2H_5$ | $C_2H_5$ | 200<br>50<br>25 | 0<br>2<br>50 | 0<br>1<br>19 | 100<br>97<br>31 | 100<br>97<br>31 |
| 306C53 | 8 | $C_2H_5$ | $C_2H_5$ | 200<br>50 | 0<br>10 | 0<br>8 | 100<br>82 | 87<br>82 |
| 344C53 | 9 | $C_2H_5$ | $C_2H_5$ | 200<br>50 | 0<br>50 | 6<br>40 | 94<br>10 | 94<br>8 |
| 402C54 | 10 | $C_2H_5$ | $C_2H_5$ | 200<br>50 | 27<br>62 | 9<br>31 | 64<br>7 | 64<br>3 |
| 598C54 | 3 | $CH_3$ | $C_2H_5$ | 50<br>25 | 47<br>56 | 41<br>35 | 12<br>9 | 12<br>4 |
| 118C54 | 4 | $CH_3$ | $C_2H_5$ | 50<br>25 | 3<br>4 | 0<br>10 | 97<br>86 | 97<br>86 |
| 198C53 | 5 | $CH_3$ | $C_2H_5$ | 50<br>25 | 0<br>5 | 3<br>5 | 97<br>90 | 97<br>90 |
| 123C53 | 6 | $CH_3$ | $C_2H_5$ | 50<br>25 | 0<br>24 | 0<br>21 | 100<br>55 | 100<br>54 |
| 395C53 | 7 | $CH_3$ | $C_2H_5$ | 50<br>25 | 0<br>0 | 0<br>5 | 100<br>95 | 100<br>95 |
| 396C53 | 8 | $CH_3$ | $C_2H_5$ | 50<br>25 | 0<br>1 | 0<br>0 | 100<br>99 | 100<br>99 |
| 397C53 | 9 | $CH_3$ | $C_2H_5$ | 50<br>25 | 8<br>56 | 2<br>26 | 90<br>18 | 90<br>15 |
| 589C54 | 3 | $n$-$C_3H_7$ | $n$-$C_3H_7$ | 200 | 64 | 21 | 15 | 5 |
| 291C54 | 4 | $n$-$C_3H_7$ | $n$-$C_3H_7$ | 200<br>50 | 45<br>55 | 10<br>38 | 45<br>7 | 45<br>0 |
| 173C53 | 5 | $n$-$C_3H_7$ | $n$-$C_3H_7$ | 200<br>50 | 60<br>77 | 40<br>23 | 0<br>0 | 0<br>0 |
| 126C53 | 6 | $n$-$C_3H_7$ | $n$-$C_3H_7$ | 200<br>50 | 33<br>54 | 27<br>39 | 40<br>7 | 39<br>0 |
| 297C53 | 7 | $n$-$C_3H_7$ | $n$-$C_3H_7$ | 200<br>50 | 48<br>49 | 28<br>45 | 24<br>6 | 23<br>0 |
| 307C53 | 8 | $n$-$C_3H_7$ | $n$-$C_3H_7$ | 200<br>50 | 56<br>67 | 41<br>31 | 3<br>2 | 0<br>0 |
| 569C54 | 3 | $C_3H_5$ | $C_3H_5$ | 200 | 68 | 30 | 2 | 0 |
| 267C54 | 4 | $C_3H_5$ | $C_3H_5$ | 200 | 21 | 27 | 52 | 52 |
| 283C54 | 5 | $C_3H_5$ | $C_3H_5$ | 200 | 19 | 33 | 48 | 45 |
| 298C53 | 6 | $C_3H_5$ | $C_3H_5$ | 200 | 64 | 31 | 5 | 0 |
| 323C53 | 7 | $C_3H_5$ | $C_3H_5$ | 200 | 66 | 26 | 8 | 7 |
| 299C53 | 8 | $C_3H_5$ | $C_3H_5$ | 200 | 63 | 35 | 2 | 0 |
| 406C53 | 7 | $CH_3$ | $n$-$C_3H_7$ | 200<br>100<br>50 | 2<br>32<br>49 | 5<br>20<br>29 | 93<br>48<br>22 | 92<br>47<br>8 |
| 590C54 | 4 | $n$-$C_4H_9$ | $n$-$C_4H_9$ | 200 | 74 | 22 | 4 | 0 |
| 300C53 | 7 | $n$-$C_4H_9$ | $n$-$C_4H_9$ | 200 | 69 | 31 | 0 | 0 |
| 308C53 | 8 | $n$-$C_4H_9$ | $n$-$C_4H_9$ | 200 | 73 | 25 | 2 | 0 |

groups were infected with *S. mansoni*, a few with *S. japonicum*. The results are shown in Table II.

*Single Oral Dose.*—The comparative activity of these three compounds when given as a single oral dose is represented graphically (Fig. 1).

A dose of 160 mg./kg. of 413C52 killed all the worms, whereas 1,000 mg./kg. of lucanthone was required to achieve the same result. With 600 mg./kg. tartar emetic 72% of the worms were killed but only 4/10 mice survived; higher doses killed all the mice and no strict comparison was possible for total worm mortality. At doses which killed all the worms 413C52 was almost 7 times as active as lucanthone. At doses which killed 72% of the worms—the maximum obtainable for tartar emetic—413C52 was 8 times as active as tartar emetic and 9 times as active as lucanthone (Fig. 1). At 160 mg./kg., 413C52

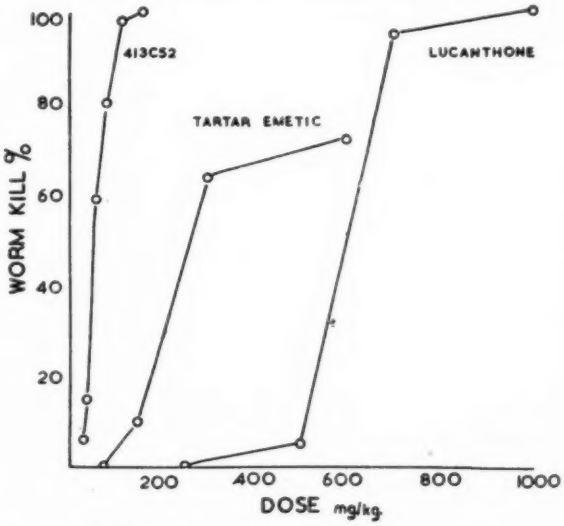



FIG. 1.—Showing the comparative activity in single oral dose of 413C52 [1: 7-bis(*p*-methylaminophenoxy)heptane], tartar emetic and lucanthone against *Schistosoma mansoni* in the mouse.

killed all the worms and was 10 times as active as tartar emetic whereas lucanthone failed to kill. However, at 250 mg./kg., lucanthone achieved the appreciable hepatic shift of 69% (Table II), but no worms were killed.

*Multiple Oral Doses.*—When mice received 10 doses in 5 days, 100% and 92% of the worms were killed with unit doses of 30 and 25 mg./kg. of 413C52, respectively (Table II). Lucanthone killed 96% of the worms at a 100 mg./kg. unit dose, that is 4 times the amount of 413C52 required to elicit a similar dose-response. When 413C52 was given in two doses of 50 mg./kg. in one day 94% of the worms were killed, compared with 96% for lucanthone at 100 mg./kg. twice daily for 5 days. Thus, 10 times as much lucanthone as 413C52 was required to obtain an approximately equal response.

Because of toxicity it was not possible to treat mice with tartar emetic above a unit dose of 40 mg./kg.; at this dose 100% hepatic shift was obtained, but the worms were alive and not ensheathed. In a group of mice similarly treated but autopsied 25 days later, 64% of the worms were in the liver but had not been killed or ensheathed. The remainder were in the mesenteric and portal veins and had, presumably, re-migrated to these vessels.

The small-scale tests with *S. japonicum* (Table II) showed that, when given twice daily for 5 days, a 50 mg./kg. unit dose of 413C52 killed all the worms; a 75 mg./kg. unit dose of lucanthone killed 26% and a 35 mg./kg. unit dose of tartar emetic produced 54% hepatic shift but did not kill.

The multiple-dose tests showed that 413C52 was much more active than either lucanthone or tartar emetic against *S. mansoni* and *S. japonicum* in mice.

### *Activity Against Other Species and Strains of Schistosome*

In addition to the standard tests against *S. mansoni* (Egyptian strain) in mice, three of the most active diaminodiphenoxyheptanes were tested orally against *S. mansoni* (Brazilian strain) in the mouse and against *S. japonicum* in the mouse, guinea-pig, and rabbit. These tests were not extensive and they were performed chiefly to determine whether the activity of the drugs extended beyond the long-established Egyptian strain of

TABLE II

COMPARATIVE ACTIVITY OF 1:7-BIS(*p*-METHYLAMINOPHENOXY)HEPTANE, LUCANTHONE AND TARTAR EMETIC ORALLY AGAINST *S. MANSONI* AND *S. JAPONICUM* IN THE MOUSE

M.V., mesenteric veins; P.V., portal vein; L., intrahepatic veins.

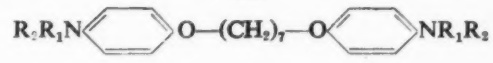
| Drug | Schistosome Species | Unit Dose (mg./kg.) | No. of Doses | Total Dose (mg./kg.) | Worm Distribution % | | | Worms Killed % |
|---|---|---|---|---|---|---|---|---|
| | | | | | M.V. | P.V. | L. | |
| 413C52 (Table I, B) | *Mansoni* | 28 | 1 | 28 | 46 | 42 | 12 | 6 |
| | | 40 | 1 | 40 | 60 | 21 | 19 | 15 |
| | | 56 | 1 | 56 | 16 | 22 | 62 | 59 |
| | | 80 | 1 | 80 | 12½ | 8 | 80 | 80 |
| | | 112 | 1 | 112 | 2 | 0 | 98 | 98 |
| | | 160 | 1 | 160 | 0 | 0 | 100 | 100 |
| | | 50 | 2 | 100 | 2 | 4 | 94 | 94 |
| | | 10 | 10 | 100 | 70 | 23 | 7 | 7 |
| | | 15 | 10 | 150 | 46 | 13 | 41 | 41 |
| | | 20 | 10 | 200 | 9 | 2 | 89 | 89 |
| | | 25 | 10 | 250 | 0 | 3 | 97 | 97 |
| | | 30 | 10 | 300 | 0 | 0 | 100 | 100 |
| | *Japonicum* | 50 | 10 | 500 | 0 | 0 | 100 | 100 |
| Lucanthone | *Mansoni* | 250 | 1 | 250 | 19 | 12 | 69 | 0 |
| | | 500 | 1 | 500 | 0 | 17 | 83 | 5 |
| | | 750 | 1 | 750 | 0 | 0 | 100 | 95 |
| | | 1,000 | 1 | 1,000 | 0 | 0 | 100 | 100 |
| | | 75 | 10 | 750 | 47 | 0 | 53 | 0 |
| | | 100 | 10 | 1,000 | 0 | 0 | 100 | 96 |
| | *Japonicum* | 75 | 10 | 750 | 58 | 16 | 26 | 26 |
| Tartar emetic | *Mansoni* | 75 | 1 | 75 | 65 | 33 | 2 | 0 |
| | | 150 | 1 | 150 | 24 | 65 | 11 | 10 |
| | | 300 | 1 | 300 | 28 | 7 | 64 | 64 |
| | | 600 | 1 | 600 | 14 | 14 | 72 | 72 |
| | | 40 | 10 | 400 | 0 | 0 | 100 | 0* |
| | *Japonicum* | 35 | 10 | 350 | 0 | 36 | 54 | 0 |

* Examination of a group of mice treated at the same time but autopsied 28 days later than this group showed 64% of the worms still in the liver but not yet dead.

TABLE III

ACTIVITY OF PRIMARY, SECONDARY, AND TERTIARY DIAMINODIPHENOXYHEPTANES AGAINST A RANGE OF SCHISTOSOME SPECIES AND STRAINS IN VARIOUS EXPERIMENTAL HOSTS

All drugs given orally either once or twice daily for 5 days or in single dose. E., Egyptian strain; B., Brazilian strain; C., Chinese strain; M.V., Mesenteric veins; P.V., Portal vein; L., Intrahepatic veins.



| Drug | Schistosome Species and Strain | Host Animal | Unit Dose (mg./kg.) | Total Dose (mg./kg.) | Worm Distribution % | | | Worms Killed % |
|---|---|---|---|---|---|---|---|---|
| | | | | | M.V. | P.V. | L. | |
| $R_1,R_2$ = H | *Mansoni* (E) | Mouse | 50 | 500 | 0 | 3 | 97 | 97 |
| | *Mansoni* (B) | ,, | 50 | 500 | 0 | 4 | 96 | 96 |
| | *Japonicum* (C) | ,, | 50 | 500 | 0 | 12 | 88 | 88 |
| $R_1$ = H $R_2$ = $CH_3$ | *Mansoni* (E) | ,, | 100 | 100 | 0 | 4 | 96 | 96 |
| | *Mansoni* (B) | ,, | 100 | 100 | 3 | 7 | 90 | 90 |
| | *Japonicum* (C) | ,, | 50 | 500 | 0 | 0 | 100 | 100 |
| $R_1,R_2$ = $CH_3$ | *Mansoni* (E) | ,, | 100 | 100 | 2 | 13 | 85 | 85 |
| | *Japonicum* (C) | ,, | 50 | 500 | 0 | 0 | 100 | 100 |
| | *Japonicum* (C) | Guinea-pig | 100 | 1,000 | 9* | 3 | 88 | 97 |
| | *Japonicum* (C) | Rabbit | 100 | 500 | 7 | 9 | 84 | 84 |

* Worms ensheathed and dead in mesenteric veins.

*S. mansoni* used for routine screening. The compounds used—the primary amine and the secondary and tertiary methylamines—were highly active against both strains of *S. mansoni* and against *S. japonicum* in a variety of rodents (Table III).

## Discussion

### *The Amino, Methylamino and Dimethylamino Series*

The first of these forms the basis for all our structural variations; the other two may be regarded as the simplest examples of monoalkyl and dialkyl substitution.

In the *primary amine* series (Table I; Fig. 2, A) at the highest unit dose (200 mg./kg.) there is full activity (100% worm mortality) over the range of values of $n$ from 3 to 9 inclusive. Maximum activity lies in the region where $n=7$ and 8, both of which compounds give over 90% mortality at 25 mg./kg. (Fig. 2).

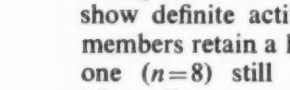
The *methylamino* series follows the same pattern as the primary amines, but there are signs of a slightly greater activity in the methylamines. For example, the compounds where $n=2$ and $n=10$ show definite activity at the highest dose; more members retain a high activity at lower doses; and one ($n=8$) still gives over 50% mortality at 15 mg./kg.

The *dimethylamino* series shows a different range of activity from any of the other series of drugs now described (Table I, C; Fig. 2, D). Whereas at a low dose (25 mg./kg.) the compound with $n=7$ is outstanding with >90% mortality, at the higher doses there is a definite alternation of activity with the number of carbon atoms in the central chain, odd-numbered members being more active than the adjacent even-numbered members (Fig. 2) This type of variation is known in certain physical properties of members of a homologous series, and the present biological results might, at first sight, seem to be related to such a phenomenon. It is true in the present series that there is an alternation of some characteristics (melting-point, solubility) with the value of $n$, but it is not confined to the dimethylamino derivatives and is found, for example, in the primary amines and monomethylamino derivatives which do not show an alternation in schistosomicidal activity.

### *The Higher Alkylamino Series*

At the two highest doses the *ethylamino* series strongly resembles the methylamino, both in absolute activity and in the variation with $n$. At 25 mg./kg., however, the results differ; a graph of activity against $n$ shows two peaks, one still at $n=7$–8 with 90–100% mortality and another at $n=4$ with nearly 80% mortality (Fig. 2, B).

The introduction of alkyl groups higher than ethyl progressively reduces the therapeutic effect. The activity of the n-*propylamino* series at 50 mg./kg. bears a strong resemblance both in absolute values and variation with $n$ to that of the ethylamino series at the dose of 25 mg./kg. The *iso-propylamino* compounds only show activity at the highest dose with virtually only the lower peak at $n=4$–5 (Fig. 2, C). At 50 mg./kg. the activity has disappeared. This difference in schistosomicidal activity between *n*- and *iso*-propylamino compounds may reflect the known difference in their chemical reactivities, for the latter are sometimes sterically hindered in reactions at the nitrogen atom. The few compounds made with *allylamino* substituents seem to resemble the *n*-propyl rather than the *iso*-propyl series.

The n-*butylamino* and iso-*butylamino* series are similar and activity is found only at the highest dose. The former shows a peak at $n=4$ and a lower peak at $n=7$, but the latter shows equal peaks at the same points. It is interesting that this branching, which is farther from the nitrogen atom  , has not had so profound an effect on the activity as with the propyl isomers.

The n-*amylamino* compounds appear to show very little activity.

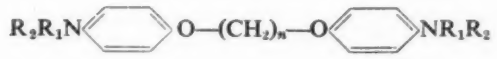
### *The Higher Dialkylamino Series*

The *ethyl-methylamino* series resembles the ethylamino and has a comparable activity. The high activity at 50 mg./kg. over the whole range ($n=4$–9) breaks down at 25 mg./kg. into two peaks, one at $n=7$–8 and the other at $n=4$–5.

The *diethylamino* series appears to be less active than the previous one, for already at 50 mg./kg. the graph of activity against $n$ produces two peaks, one at $n=7$–8 and the other at $n=4$.

Both the *di*-n-*propylamino* and the *diallylamino* series show a low level of activity, and the *di*-n-*butylamino* compounds are inactive.

Comparing the monoalkyl with the dialkyl series, it is apparent that, for a given *total* of carbon atoms present as alkyl substituents, a dialkyl is in general more active than a monoalkyl compound. For, although dimethyl is approximately equal to ethyl, the following inequalities have been observed: ethylmethyl>*n*-propyl; diethyl>*n*-butyl or *iso*-butyl; and, in a single comparison, methyl-*n*-propyl>*n*-butyl.



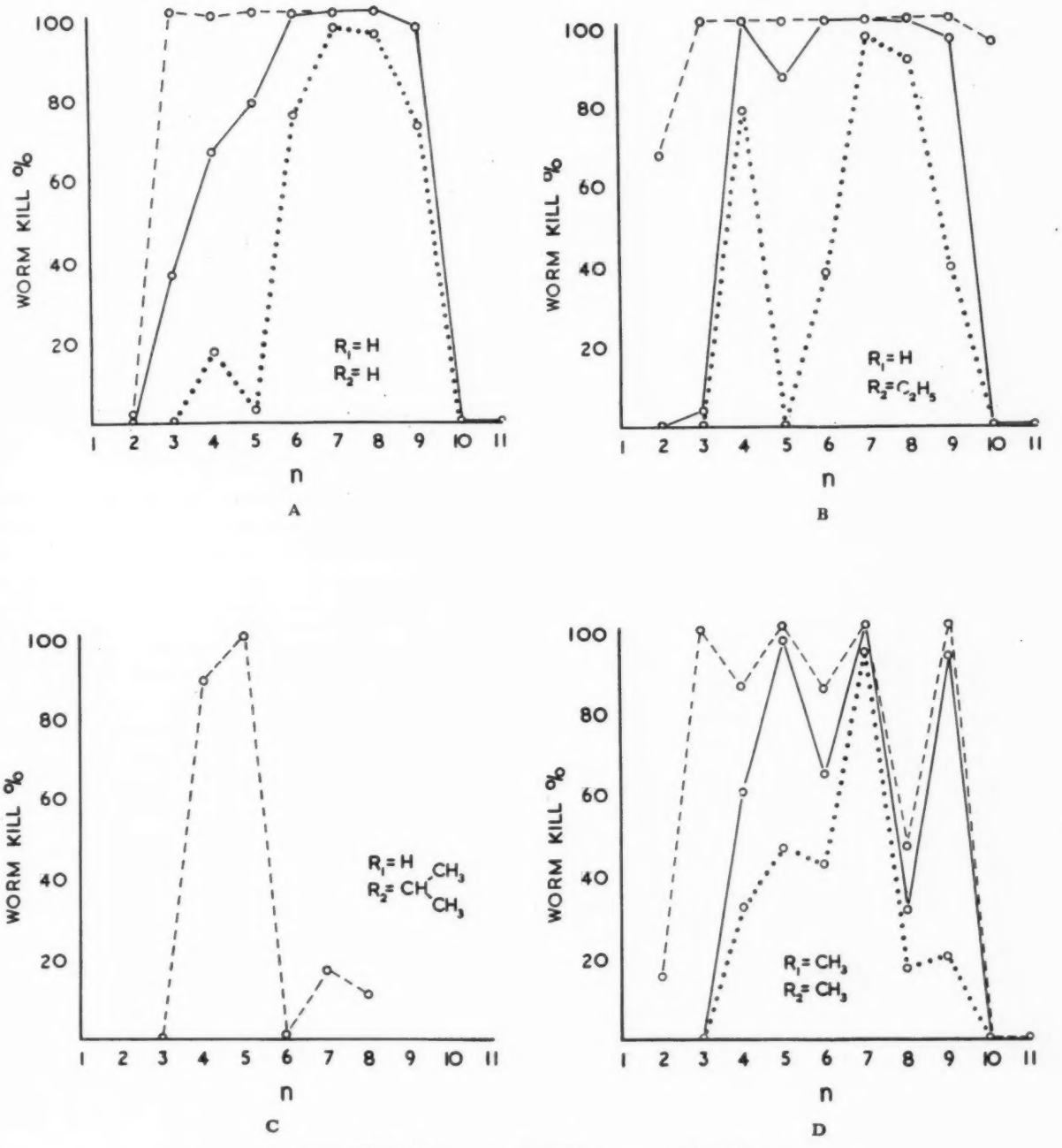




FIG. 2.—Showing the four types of relationship between structure and schistosomicidal activity of diaminodiphenoxyalkanes.

Fig. 2 illustrates the four types of relationship between structure and schistosomicidal activity which have been observed in these compounds.

It is too early yet to attempt a detailed explanation of our results, but the variations of *in vivo* activity with substitution on the nitrogen atom and with variation in the methylene bridge suggest a superposition of effects. In elaborating the primary amines in the manner described, the picture obtained by plotting activity against $n$ changes its form at the point ($NHC_2H_5$ in monosubstitution and $N(CH_3)_2$ in disubstitution) where two carbon atoms have been added to each nitrogen atom. Apart from the unique dimethylamino series, the change is from a single peak at $n=7$–8 to double peaks at $n=4$–5 and $n=7$–8, followed then by a relative accentuation of the lower peak as the substitution on the nitrogen atom increases. It is possible at this stage to envisage these *in vivo* results as representing a summation of intrinsic schistosomicidal activity and some property which determines the absorption of the compounds in the host. It is hoped that further detailed studies will help to elucidate these points.

*The Effect of Drug Treatment*

All active schistosomicides when administered to infected animals affect the worms in more or less the same way. The worms in mesenteric and portal veins are rendered unable to maintain their position in the vessels; they are swept back passively by the portal blood, and accumulate in the liver, where they are surrounded by inflammatory tissue and then destroyed by phagocytosis. This sequence of events has been followed in detail in mice treated with lucanthone and trivalent antimonials (Standen, 1953): unless treatment is prolonged, or unless dosage is sufficiently high to produce immobility of the worms for sufficient time, the worms recover and return to the mesenteric veins. By comparison, the diaminodiphenoxyalkanes produce a novel effect. The degree of hepatic shift is proportional to the amount of drug given and is also related to the absolute activity of any given compound. At any dose producing some degree of hepatic shift, even with compounds of relatively low activity, all worms which drift back to the liver become ensheathed in inflammatory tissue within the liver and are subsequently destroyed by phagocytosis. With lucanthone and the trivalent antimonials a low degree of hepatic shift is invariably followed by remigration to the mesenteric veins. It appears that the effect of the new compounds is irreversible. Therefore, although the mode of action of the diaminodiphenoxyalkanes conforms in most ways to that of schistosomicides in general, this fundamental and irreversible effect constitutes an outstanding difference. Because of this it has been possible to obtain controlled dose-response in terms of worm mortality rather than hepatic shift, and thus to provide a more accurate measure of drug activity.

A detailed account of the process of worm destruction in the liver will appear elsewhere, but it can be recorded here that these compounds are not themselves specifically schistosomicidal but that they prepare the parasites for invasion by phagocytes. The onset of phagocytosis is gradual, invasion commencing where the cuticle of the worm is in contact with the wall of the intrahepatic vein and then proceeding to affect the worm throughout its length. In one experiment ensheathed and phagocytosed worms were observed in the mesenteric veins of a treated guinea-pig infected with *S. japonicum* (Table III). This is atypical and is alone amongst many thousand observations where phagocytosis was seen to occur only in the liver.

*Conclusions*

Symmetrical diaminodiphenoxyalkanes having the amino-groups in the *para* positions are highly active schistosomicides. Altering the position of the amino-groups or quaternization destroys this activity. Within the active series, considerable variation of alkyl substitution on the nitrogen and of the methylene bridge is possible while still retaining activity. A detailed investigation of the influence of these changes on biological activity has been made, but at present any attempt to account for these findings can be only speculative. It appears likely that a final appraisement must take into account the dual need for absorption by both host and parasite.

Against *S. mansoni* in mice the outstanding differences between these compounds and lucanthone or the antimonials are the very high activity at low dosage and the more potent effect upon the parasites. Also, although the experiments performed with *S. japonicum* have not been extensive, sufficient evidence of activity against this parasite is available to justify the conclusion that the schistosomicidal action of the diaminodiphenoxyalkanes is not species specific.

## Summary

1. High schistosomicidal activity has been discovered in symmetrical $p:p'$-diaminodiphenoxyalkanes when tested orally against *S. mansoni* and *S. japonicum* in mice, guinea-pigs, and rabbits.

2. The influence on activity of alkyl substitution in the amino-groups and of variation in the methylene bridge has been investigated in detail.

3. The primary amines and mono-methylamines show a peak of activity at a chain length ($n$) of 7–8. The dimethylamines are unique in showing an alternation of activity with $n$, but still show a peak where $n=7$. All the remaining mono- and di-alkylamines ($C_2$–$C_5$) show peaks at $n=4$ and $n=7$–8 and in general show decreasing activity as the size of alkyl group increases.

4. The most active of the diaminodiphenoxyalkanes are several times as active as lucanthone or tartar emetic when given orally to mice infected with *S. mansoni* or *S. japonicum*.

We are indebted to Dr. A. G. Caldwell, Wellcome Research Laboratories, and to Mr. T. M. Sharp, Chief Chemist, Wellcome Laboratories of Tropical Medicine, for the preparation of certain compounds mentioned in this paper. We wish to record that the work described would have been impossible but for the valuable and loyal assistance of our technical staffs, and we extend to them our most grateful appreciation.

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# A SLOW CONTRACTING SUBSTANCE IN NORMAL HUMAN URINE

BY

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Crude urine of man and other animals as well as urinary extracts have repeatedly been shown to have a stimulating effect on isolated plain muscle. However, few attempts have been made to characterize and purify the active principles.

As it had been observed that superfused rat's uterus showed great sensitivity to diluted human urine (Gaddum, unpublished), a study of the properties of the substance causing this effect was undertaken. Besides being extremely sensitive, this preparation has the advantage of requiring only small volumes of active solutions.

## METHODS

*Rat's Uterus.*—Uteri from virgin rats weighing 160–190 g. were superfused with Jalon's solution (Gaddum, Peart, and Vogt, 1949) by the technique described by Gaddum (1953a). At first they were injected the day before the experiment with stilboestrol (10 $\mu$g./100 g. wt.), but later the use of stilboestrol was abandoned because the uterus in artificial oestrus, though usually more sensitive, also frequently gave irregular responses.

Only freshly voided urine of normal people of both sexes was used and no considerable individual variations were observed. The preparation responds usually to a concentration of 1% (v/v) urine, though, sometimes, depending on the sensitivity of the organ and the activity of urine, responses are elicited with dilutions as low as 0.1%. Only exceptionally was it necessary to use concentrations higher than 2% to get suitable contractions.

Dilutions of urine and its extracts were made in the superfusion fluid, to which atropine sulphate (1 mg./l.) was routinely added. Of these dilutions 5 drops were applied to the uterus, after stopping the flow, and allowed to act for 30 sec. before being washed away. The contractions show a characteristic latent period variable with the dose, and greater in the less active samples. They are quite different from those induced by acetylcholine or 5-hydroxytryptamine and similar to those obtained by bradykinin, angiotonin, or substance P. They begin usually 20–40 sec. after the addition of the solutions and reach the maximum after 60–100 sec.

The administrations were made at intervals of 4 or 5 min., the latter interval generally giving more uniform responses.

A good preparation discriminates between doses differing by 20%. Tachyphylaxis for the active substance studied has not been observed; on the contrary, as a rule, there is a progressive sensitization during the first stages of the experiment. That the organ in superfusion is maintained in a good condition is shown by the fact that it was often possible to work with the same uterine horn for more than 8 hr.; sometimes the same horn was even used for 2 consecutive days, being kept in the refrigerator overnight.

Experiments done with the urine of different animals (rat, cat, dog, rabbit, guinea-pig), withdrawn from the bladder immediately after death, showed the same property of stimulating the rat's uterus, in the characteristic slow-reacting way. No attempt was made to identify the responsible principle (or principles) with that of human urine, which has been exclusively used in the present experiments.

*Guinea-pig's Ileum.*—From animals weighing 200–250 g., fasted for 20 hr., a piece of terminal ileum was removed and superfused with Tyrode's solution containing atropine sulphate ($10^{-6}$) and mepyramine maleate ($10^{-6}$). In this preparation, as pointed out by Gaddum (1953a), it was necessary to shorten the stoppage of flow to 15 sec.

*Hen's Rectal Caecum.*—This organ was also superfused with Tyrode's solution containing atropine and mepyramine as above. Owing to slow relaxation the intervals between application were 6–7 min. Stoppage of the flow for 30 sec. did not cause contraction.

*Blood Pressure in the Rat.*—The technique as described by Dekanski (1954) for the assay of angiotonin was followed. All the drugs were tested before and after dibenamine treatment.

*Blood Pressure in the Rabbit.*—Rabbits (1.8–2.3 kg.) under urethane anaesthesia (8 ml. of a 20% sol. i.v.) were used. Heparin (1,000 units of heparin "Boots") was injected routinely. Arterial blood pressure was recorded from the carotid artery by a Hg manometer and drugs were injected in the external jugular vein at 5–10 min. intervals. Assays were made both before and after section of the vagus.

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*Chemicals.*—The substance P was a preparation made by A. H. Amin and T. B. B. Crawford and estimated to contain 13.8 Euler units/mg. The bradykinin was a preparation obtained from M. Rocha e Silva through H. O. Schild. The angiotonin was "solution angiotonin" containing 10 cat units/ml., made by Eli Lilly and Co., and kindly supplied by J. Dekanski.

Lysergic acid diethylamide was kindly presented by Sandoz Products and 5-hydroxytryptamine creatinine sulphate by Messrs. Upjohn.

Crystalline preparations of trypsin, chrymotrypsin, and pepsin were obtained from Armour and Co., Ltd.

## Results

### *Extraction*

It was found that both activated charcoal (B.D.H. 10 g./l.) and fuller's earth (2 g./l.) adsorbed the active principle of urine. In these concentrations they retained at least 90% of the activity when shaken with urine for 2 to 4 hours.

*Method A.*—A first method of extraction was developed using charcoal and eluting with glacial acetic acid (5 ml./g. of charcoal). It was found that the recovery in this stage was increased by bringing the urine to *p*H 9. The addition of 10 volumes of ether to the eluate led to the quantitative precipitation of the active material. A further purification consisted in the addition of ethanol with 20% water to the precipitate, in which the active principle dissolved, leaving a considerable amount of insoluble and inactive material. The supernatant fluid was reduced in volume in a vacuum at 40° C. and afterwards completely dried in high vacuum in the presence of $P_2O_5$. The resulting product was a very hygroscopic yellow powder. The final recovery, with this method, did not exceed 30% owing to difficulties in eluting the active substance from the charcoal, even when acetic acid was repeatedly added.

*Method B.*—A modification of the method employed by Clark, Winckler, Gollan, and Fox (1954) for the extraction of angiotonin was found to be simpler and more advantageous. Urine is acidified with HCl to *p*H 1.5, saturated with NaCl, and extracted by shaking with *n*-butanol. With Clark's procedure (extraction three times with 0.25 vol.) only about 60% was recovered (30% in the first extract). When an equal volume of butanol was used in a single extraction the recovery of the oxytocic activity was practically 100%. The final product obtained in this way was more active and more soluble in water than that obtained by repeated extractions with smaller volumes. Both conditions—acidification and saline saturation—were seen to be indispensable for the passage of the active principle to the butanol.

The butanol layer was separated by centrifugation. The addition of 5 vol. of ethyl ether to this precipitated the active material. This precipitation was as complete at room temperature as at −20° C., which those authors consider necessary for angiotonin. After drying *in vacuo* at 40° C. a pinkish white powder was obtained, 1 litre of urine yielding about 1 g. This preparation is stable and appears to contain all the active substance originally present. Further purification has not yet been attempted.

### *Dialysis*

The active principle is slowly dialysable through cellophane. In experiments with urine against distilled water, using dialysers similar to those described by Verney (1926) and mounted in a shaker, equilibrium was slowly reached. After 4 hr. the activity in the water was practically nil and there was no appreciable loss in the urine. After 20 hr. the activity was equally distributed on both sides.

### *Stability*

The stability of the substance in the urine and of the extracts in aqueous solutions depends on the *p*H. After 24 hr. at room temperature there was a loss of activity of about 50% in different urines at *p*H 7. In one sample of urine brought to *p*H 5 with 4N-HCl, and kept in the same conditions, the destruction did not exceed 10–20%, whereas in another sample of the same urine at *p*H 9 (4N-NaOH was added) more than 75% was destroyed in the same period of time. Aqueous solutions of extracts gave similar results.

A solution of a butanol extract kept in the refrigerator (4°) at *p*H 5 showed, after a fortnight, a 10 to 20% loss of activity.

The thermostability was studied with extracts made by the second method described. Samples of solutions in 0.03N, 0.3N, and 1N-HCl and 0.1N-NaOH were placed in boiling water in test-tubes closed with rubber dam and specimens for assay taken at different intervals. In 0.03N-HCl practically all the original activity was present after 6 hr. In 0.3N-HCl 50% was destroyed at the end of 1 hr. and 75% after 4 hr. boiling. More than 80% was inactivated after 30 min., when N-HCl was used as solvent. In alkaline solutions the substance is much less stable. There was a loss of 50% after 15 min. and complete destruction after 30 min. when it was boiled in 0.1N-NaOH.

At a *p*H near neutrality urine stands boiling for periods as long as 4 hr. without apparent loss of activity.

*Solubility*

The active principle is soluble in water, glacial acetic acid, aqueous ethanol (80%), aqueous acetone (80%), phenol, *n*-butanol and methanol. It is completely insoluble in ether, chloroform, benzene and petroleum ether, scarcely soluble in absolute ethanol, and practically insoluble in pure acetone.

*Action of Proteolytic Enzymes*

Both urine and solutions of butanol extracts were completely inactivated by chymotrypsin after incubation at *p*H 8 and 38° C. for 60 min. (Fig. 1*a*). Neither trypsin nor pepsin caused any change in the activity when incubated for 4 hr. at *p*H 8 and 2, respectively (Fig. 1*b* and 1*c*). The powerful stimulating activity of trypsin for rat's uterus made it difficult to study the action of this enzyme. Contrary to what had been described for guinea-pig's ileum (Rocha e Silva, 1951) the organ was not desensitized after repeated administrations of the enzyme. Making use of the greater thermostability of the urinary principle it was possible to destroy trypsin almost completely, by boiling for 30 min., and prove the preservation of that principle in the incubate.

*Paper Chromatography*

Extracts obtained by both methods were studied by paper chromatography, using the ascending method in Whatman No. 1 filter paper, washed previously with 0.01N-HCl. The solvents were the organic phase of the mixture *n*-butanol-acetic acid-water (4 : 1 : 5) (Partridge, 1948) and phenol saturated with 0.01N-HCl. The chromatograms were developed at room temperature (15–18° C.) for 20–24 hr., in most of the experiments in air, in only a few in nitrogen which proved to be unnecessary.

The localization of the active substance was detected by biological assay. Strips of paper were cut at variable intervals from the starting line to the solvent front, and eluted by descending chromatography, overnight, in small individual troughs (7 ml. capacity) with 0.01N-HCl in an airtight chamber. The eluates were evaporated to dryness in a vacuum (40° C.), taken up in Jalon's solution and assayed on the superfused uterus.

With the purest extracts obtained by the first method there were always at least three spots stained by ninhydrin (0.2% solution in acetone with 5% acetic acid). These bore no relation to the active principle, since their $R_F$ was much lower

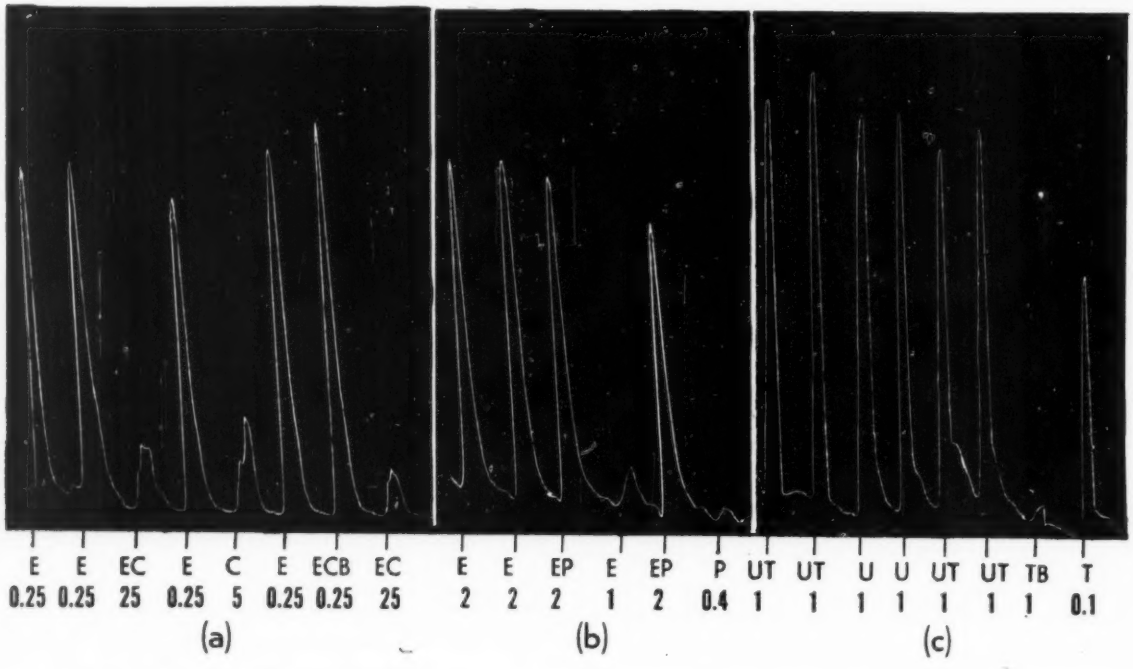


FIG. 1.—Rat's uterus. Superfusion. Active substance destroyed by chymotrypsin, but not by pepsin or trypsin. Concentrations mg./100 ml. (solids); ml./100 ml. (urine). E, dried extract of urine. C, chymotrypsin. P, pepsin. T, trypsin. TB, trypsin boiled 30 min. EC, E incubated 1 hr. with chymotrypsin (0.2 mg./mg. E). ECB, control with boiled chymotrypsin. EP, E incubated 4 hr. with pepsin (0.2 mg./mg. E). U, urine boiled 30 min. UT, urine incubated 4 hr. with trypsin (10 mg./ml.) and then boiled 30 min.

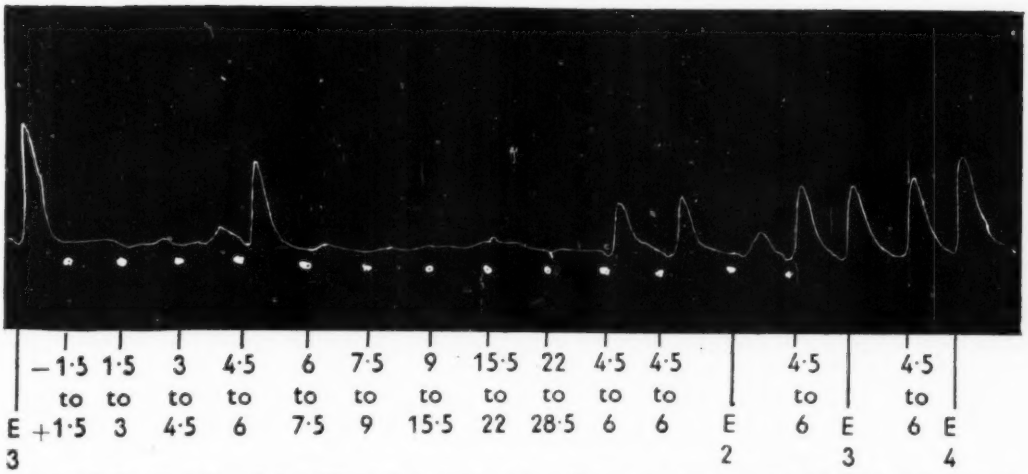


FIG. 2.—Rat's uterus. Superfusion. Effects of eluates from ascending paper chromatogram developed with butanol-acetic acid-water for 24 hr. Distances from starting line in cm. Solvent front 28 cm. E, butanol extract of urine (mg./100 ml.). Weight of E used, 0.4 mg. Volume of eluates 4 ml. $R_F$, 0.18. Total recovery 30%.

than that of the biologically active strip in chromatograms developed with phenol.

The butanol extracts, in the amounts used (spots containing 0.2 mg. of extract), did not give any coloured spot with ninhydrin or with Pauly's reagent.

The $R_F$ values obtained by biological assay showed small variations with the purity of the product and were about 0.2 in the butanol-acetic acid-water mixture and 0.9 in phenol. With both solvents the total recovery from the paper chromatogram was about 30%, all the activity being always confined to a narrow zone (Fig. 2).

One of the butanol extracts was submitted to acid hydrolysis for chromatographic study. 0.5 ml. of 4N-HCl was added to 50 mg. of extract in an ampoule that, after being sealed, was put in an oven at 100° C. for 24 hr. The contents of the ampoule were afterwards dried on a boiling-water bath *in vacuo* and redissolved in 5 ml. of distilled

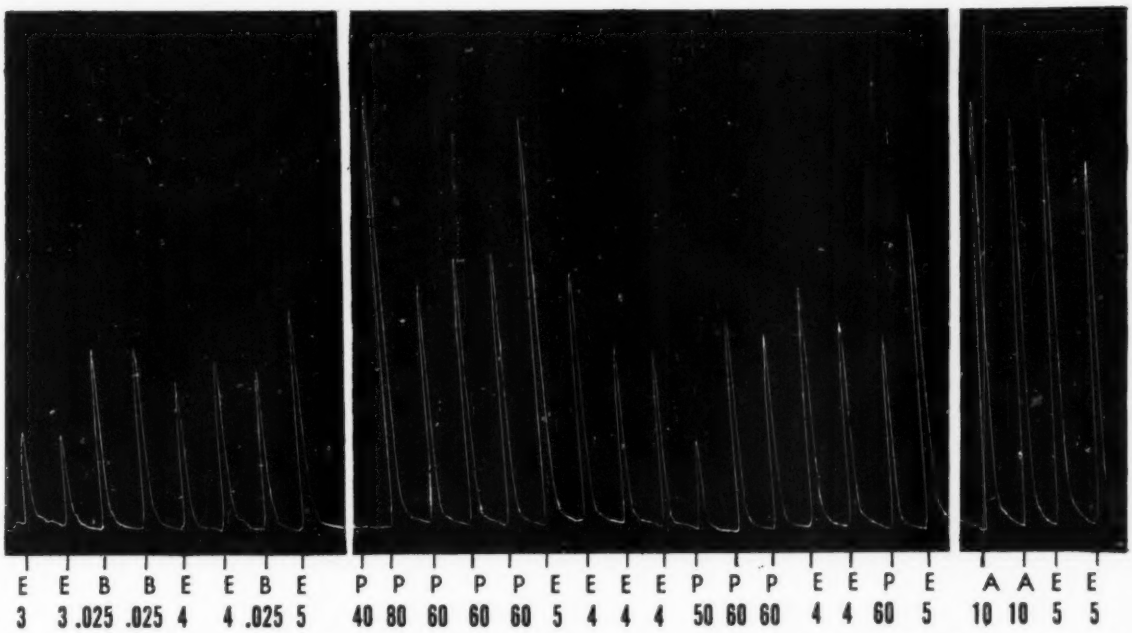


FIG. 3.—Rat's uterus. Superfusion. Comparison between a butanol extract of urine (E), bradykinin (B), substance P (P), and angiotonin (A). Concentrations in mg./100 ml. (E and B) and units/100 ml. (P and A).

water. After filtration the hydrolysate was applied to filter paper (spots of 0.02 ml.) and developed by ascending chromatography with both of the above mentioned solvents. In these conditions there were at least 6 ninhydrin positive spots which have not yet been identified. A solution of the original extract, in the same concentration and run simultaneously, failed to show any spot.

*Other Pharmacodynamic Properties*

Besides superfused rat's uterus (Fig. 3) a few other preparations have been used to distinguish the active principle under study from other known substances with similar properties. Parallel assays were made using the guinea-pig's ileum, hen's rectal caecum and rat's or rabbit's blood pressure with substance P, bradykinin and angiotonin. Bradykinin could not be used for blood-pressure assays, as it was not available in sufficient amount.

Both urine and urinary extracts were less active on the guinea-pig's ileum than on the rat's uterus. In some experiments concentrations as high as 10% of urine only elicited small contractions in the ileum. Furthermore, this organ did not give, as did rat's uterus, regular responses consistent with an accurate estimation of activity. As a rule the contractions were in a "staircase," very different from those in the uterus, with a latent period of 40–60 sec.

No contraction was obtained in the hen's rectal caecum, though very high concentrations of urinary extract were used. Bradykinin, substance P and angiotonin elicited contractions of this preparation as reported by others.

The rat's blood-pressure preparation, in the conditions used, was very sensitive to angiotonin, but gave weak and irregular responses to substance P, as was found by Amin, Crawford and Gaddum (1954). The urinary extracts elicited irregular responses, and clear hypotensive effects were only observed after the injection of large doses, and were then accompanied by symptoms of general intoxication followed after a short period by the animal's death.

In the rabbit too it was necessary to administer relatively high doses to obtain depressor effects and these were far from constant. Some animals seemed particularly resistant, and in the same animal an originally hypotensive dose produced no effect when repeated some time later. Comparison with substance P (Fig. 4) in doses equiactive on the uterus, or even on the ileum, showed that the depressor action of the urinary extracts was feeble.

In a rabbit that gave clear hypotensive responses the comparison was made of a butanol extract and the urine used in its preparation. It was found (Fig. 4) that the hypotension produced by the extract, in a dose corresponding to 100 times that of urine, was of a much shorter duration.

Since the oxytocic substance was quantitatively extracted in other experiments by butanol, the depressor effect of the urine must be mainly due to other substances.

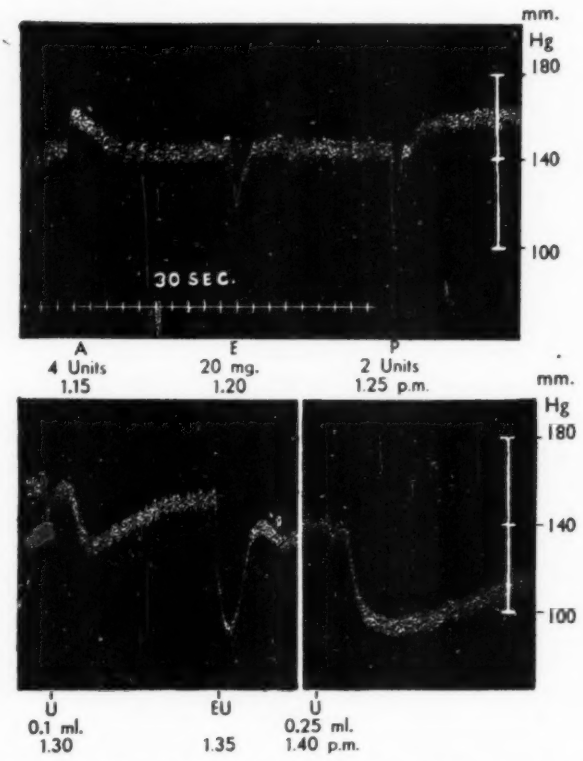


FIG. 4.—Rabbit. 2.3 kg. Arterial pressure. Urethane. Vagi cut. Artificial respiration. Intravenous injections. A, angiotonin. E, same extract as in Fig. 3. P, substance P. U, urine. EU, butanol extract from 25 ml. of the same urine.

Table I shows the results of assays of a urinary extract against substance P, angiotonin and bradykinin by several different methods. It will be seen that when substance P or angiotonin was used as a standard the urinary extract showed high activity on the rat's uterus compared with much lower activity on the other tissues used. The active sub-

TABLE I

PARALLEL ASSAYS OF THE AMOUNTS OF OTHER POLYPEPTIDES EQUIVALENT TO 1 MG. OF A PREPARATION OBTAINED FROM URINE

| | | Substance P (Units) | Angiotonin (Units) | Bradykinin (mg.) |
|---|---|---|---|---|
| Rat .. | Uterus | 15 | 2 | 0·006 |
| Guinea-pig | Ileum | 0·6 | 0·16 | 0·008 |
| Hen .. | Rectal caecum | $<0·08$ | $<0·08$ | $<0·08$ |
| Rabbit .. | Blood pressure | $<0·1$ | Pressor | Not done |

stance cannot therefore be substance P or angiotonin. On the other hand, when bradykinin was used as a standard the results obtained with rat's uterus and guinea-pig's ileum agreed well with one another, and the results obtained with hen's rectal caecum were consistent with the theory that the active substance is identical with bradykinin. The urinary extract had no effect on this preparation in the doses used, but the other preparations all had some effect, and it was found that roughly 1 unit of substance P, 1 unit of angiotonin and 1 mg. of the preparation of bradykinin were equivalent to one another in their actions on the hen's rectal caecum.

## Discussion

The inactivation by chymotrypsin, the slow dialysis, and the chromatographic behaviour suggest that the active principle under study is a polypeptide. The fact that it can be extracted by methods used for the extraction and purification of substances of established polypeptide nature supports this view.

The complete suppression of the stimulating activity of urine by chymotrypsin and by boiling in strong acid or alkaline media, and the localization of all the activity in a single zone of chromatograms developed in two different solvents, suggest that a single substance is responsible for the effect on rat's uterus.

However, other substances present in the urine could contribute to the stimulating action. Therefore some experiments were made in order to clarify this point.

Urea and potassium have no action on the uterus in concentrations much higher than those likely to be present in the diluted urines used in these experiments.

Nicotine, which has been found in the urine of smokers (Helmer, Kohlstaedt, and Page, 1939), had no action on the uterus in concentrations from 0.05 mg. to 100 mg./ml. of the tartrate, nor did it modify the response to extracts of urine if simultaneously added.

Work done by Twarog and Page (1953) with the isolated heart of *Venus mercenaria* led to the conclusion that 5-hydroxytryptamine (HT) is normally present in human urine. Though the rat's uterus in superfusion is very sensitive to this substance, the concentrations mentioned by those authors are below the threshold of the preparation. Making use of the inhibitory action of the lysergic acid diethylamide (LSD) (Gaddum, 1953b) it was possible to demonstrate persistence of the response of the organ after the addition of urine and complete abolition of the effects of a solution of HT which was equipotent before the addition of that antagonist (Fig. 5).

Darmstoff was originally assumed by Kuck and Vogt (1950) to be identical with substance P, but is distinguished from this and the urinary principle by its solubility in chloroform.

Kallikrein (Frey, Kraut, and Werle, 1950) is easily distinguished because it is thermolabile and undialysable, and does not contract the rat's uterus or the guinea-pig's ileum.

Wollheim's Depressan is a substance which, according to the author (Wollheim, 1937), is present in the urine of normotensive individuals and absent in those suffering from essential hypertension. It is a potent depressor and it has, in its purified preparations, practically no stimulating action on the intestine or uterus. Other properties add to its distinction: it is not dialysable, it is not adsorbed by charcoal, it is destroyed by pepsin and is less thermostable than the principle discussed here.

A similar if not identical depressor substance was described by Little, Green, and Bumgarner (1948). Stewart (1953) reported the stimulating effect of extracts of human urine on the isolated uterus of the guinea-pig or mouse, and on the guinea-pig's ileum, the latter organ being less sensitive. This active principle is stated to be undialysable through cellophane.

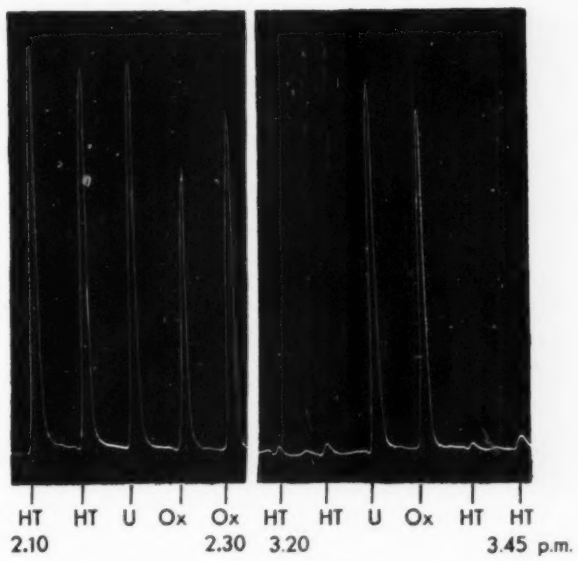


FIG. 5.—Rat's uterus. Superfusion. HT, 5-hydroxytryptamine 10 $\mu$g./l. U, urine 1%. Ox, oxytocin 0.3 units/l. Lysergic acid diethylamide ($10^{-7}$) in the superfusion fluid from 2.35 onwards. The effect of HT was abolished, but not that of U or of Ox.

Although the same activity has been found in female urine, the possibility of contamination of male urine, which was mostly employed, with semen must be considered. A depressor and plain muscle stimulating substance in human semen has been reported independently by Goldblatt (1935) and v. Euler (1936a). This secretion was shown to induce marked contraction of the uterus of different animal species, including the rat. The active principle, according to Euler, is easily distinguishable from the urinary substance by its solubility in organic solvents (ether, chloroform) and its strong and lasting hypotensive effect.

It remains to discuss the relation of the substance described here to a group of natural polypeptides which stimulate plain muscle and are described in a book edited by Gaddum (1955).

There is evidence that posterior pituitary hormones are excreted in the urine. It will be seen that the oxytocic action of the urine used in the experiment shown in Fig. 6 was equivalent to that of 40 units of oxytocin/l. This effect was not, however, due to the presence of these large amounts of oxytocin, since treatment of the urine with thioglycollate by the method of Ames, Moore, and van Dyke (1950) did not inactivate it.

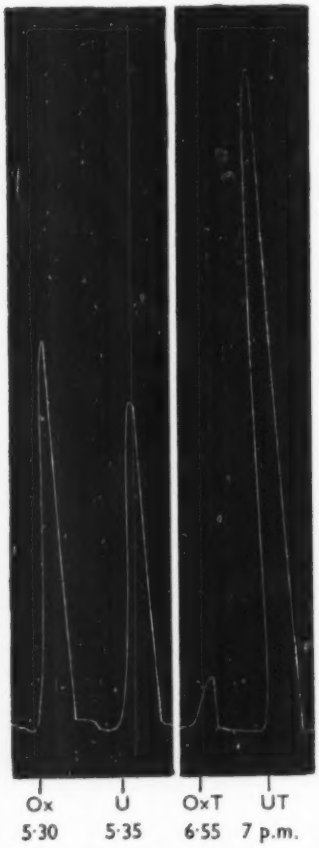


FIG. 6.—Rat's uterus. Superfusion. Ox, oxytocin 0.3 units/l. U, urine (1%). OxT and UT, after treatment with thioglycollate which inactivated Ox but not U.

Table I shows that the active substance is not substance P (Euler and Gaddum, 1931; Gaddum and Schild, 1934). The fact that substance P is inactivated by trypsin (Euler, 1936b) is also evidence against this identification.

The fact that the rat's uterus is, according to Ludueña (1940), particularly sensitive to angiotonin (hypertensin) as well as many of its physical and chemical properties suggested that this might be the substance in question. It was for this reason that Clark's method of extraction was used. The results shown in Table I, together with the fact that angiotonin is inactivated by pepsin and trypsin, seemed to disprove this view. None of these urinary extracts had a pressor action.

Bradykinin (Rocha e Silva, 1951) shows many similarities with the urinary principle (dialysis, stability at different *p*H's, solubility, action of proteolytic enzymes, etc.). The results shown in Table I are in favour of their identity, but more evidence is required.

The effects described here seem to be due to a substance, or group of substances, which have been studied by various groups of workers. Kallidin was described by Werle (1937) and given its name by Werle and Berek (1950). It is formed by the action of an enzyme in urine on blood. According to Werle, Kehl, and Koebke (1950) it is identical with bradykinin (Rocha e Silva, Beraldo, and Rosenfeld, 1949), which is formed by the action of snake venom or trypsin. Substance U (Beraldo, 1952, 1955) is formed by urine in much the same way that kallidin is formed, and substance Z (Werle and Erdös, 1954) is naturally present in urine. All these substances share with the substance described here the property of being destroyed by chymotrypsin, but not by trypsin.

The main value of the present work is thought to lie in the fact that it provides a simple method of assay which measures activity of this kind and which does not appear to be significantly affected by any of the other known active principles which may be present in urine. It also provides a method of making a dry standard preparation.

## SUMMARY

1. Normal urine diluted 1:100 has been found to cause contraction of superfused rat uterus.

2. The active principle is stable in weak acids, dialysable, and soluble in 80% aqueous acetone or ethanol, but not in ether or chloroform. It was destroyed by a preparation of chymotrypsin, but not by trypsin or pepsin, and is probably a polypeptide. Two methods are described by which active dry preparations of the substance may be made.

3. Evidence is presented that this effect is not due to potassium, urea, nicotine, 5-hydroxytryptamine, kallikrein, depressan, oxytocin, substance P or angiotonin.

4. The active principle is closely allied to the substance, or substances, known as kallidin, bradykinin, substance U and substance Z.

This research was carried out at the suggestion of Professor J. H. Gaddum, to whom I wish to express my gratitude for advice and criticism throughout it.

The work was done during the tenure of a British Council Scholarship.

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# THE MODE OF ACTION OF SULPHONAMIDES, PROGUANIL AND PYRIMETHAMINE ON *PLASMODIUM GALLINACEUM*

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Despite the mass of information on the antimalarial action of sulphonamides, proguanil, and pyrimethamine (see Goodwin and Rollo, 1955), there is as yet no complete picture of the relationship between them, although they all probably act upon the same metabolic pathway in the synthesis of nucleoprotein. Hawking (1953a) in his review of protozoal chemotherapy pointed out: "Many different lines of work appear to be converging here towards a general explanation, but it will be necessary to achieve further elucidation of the metabolism of *p*-aminobenzoic acid (PAB) and of folic acid by the malarial parasite before all the different facts in the jigsaw puzzle can be fitted into place." In this paper an attempt is made to fill some of the gaps in our knowledge of cross-resistance, potentiation, and antagonism between antimalarial drugs, by reviewing and analysing the known facts, in the light of new data.

## METHODS

The parent strain of *Plasmodium gallinaceum* was that maintained in these laboratories for many years by blood and occasional mosquito passage in young chicks. This and the other drug-treated strains have, during the course of the experiments, been passaged solely by blood inoculation. Five- or twelve-day-old chicks (Rhode Island Red–Light Sussex cross) were inoculated intravenously with approximately 50 million parasitized red blood cells. The antimalarial drugs were given orally either in solution or, if insoluble in water, in gum tragacanth suspension. Starting a few hours after inoculation, a total of seven doses was given over 3½ days. Infection was assessed from stained blood films on the fourth day after inoculation, when in untreated controls about 70–90% of the red blood cells were infected. The infected red cells in the test animals were counted and the results were expressed as percentages of the controls. That dose which reduces parasitaemia to 50% of the mean parasitaemia of untreated controls (ED50) was obtained from a 3- or 4-dose assay (Rollo, 1952). A group of five chicks was normally used at each dose level.

*Cross-resistance.*—A strain of *P. gallinaceum* (P.36), which was highly resistant to proguanil and to pyrimethamine, was obtained from Dr. D. G. Davey, of Imperial Chemical Industries. The sensitivity of this strain to sulphadiazine was tested in order to complete the picture of cross-resistance relationships reviewed by Thurston (1953). Two further strains were prepared by treating successive passages with subcurative doses of proguanil and pyrimethamine respectively. During each passage the chicks received a total of seven doses of the drug as described above. Both strains were passaged and treated in parallel and were tested periodically for cross-resistance during the early stages of the development of drug resistance.

*Potentiation.*—The potentiating effect of pyrimethamine upon the activity of proguanil was investigated by giving the drugs both singly, and together in various proportions, to groups of infected chicks. ED50's were determined from the dose-response curves and were plotted on a graph to demonstrate the effect of one drug upon the action of the other. Similar experiments were done using pyrimethamine with sulphadiazine, sulphaguanidine, succinylsulphathiazole, penicillin, or streptomycin. In some experiments the blood levels of the sulphonamides produced by single oral doses were determined by the method of Bratton and Marshall (1939).

*Inhibition.*—The effects of PAB and folic acid on the activity of sulphadiazine and pyrimethamine were determined. The PAB or folic acid was given intraperitoneally in aqueous solution or suspension 30 min. before each oral dose of the antimalarial drug except in one experiment with pyrimethamine in which PAB was given orally five times a day at three-hourly intervals; in addition, the chicks were fed on a diet containing 0.1% PAB. To ensure that the diet would be consumed at night when the chicks were not being dosed, the cages were darkened throughout the day and brightly lit during the night.

The inhibitory action of amino-an-fol (2, 4-diaminopteroylaspartic acid) was investigated in one experiment.

## RESULTS

*Cross-resistance.*—The effect of sulphadiazine upon strain P.63 is shown in Table I. There was no evidence of resistance to sulphadiazine although this strain was highly resistant both to proguanil

and to pyrimethamine. Indeed, there is some evidence of hypersensitivity to sulphadiazine.

The pattern of development of resistance in the two other strains which had been treated in parallel with proguanil or pyrimethamine is shown in Table II.

TABLE I

THE ACTION OF SULPHADIAZINE ON NORMAL AND PROGUANIL-PYRIMETHAMINE RESISTANT STRAINS OF *P. GALLINACEUM*

| Strain | Drug | Dose (mg./kg.) | Parasitaemia* (%) |
|---|---|---|---|
| Normal .. | Proguanil | 2·0 | 12 |
| | Pyrimethamine | 0·06 | <1 |
| | | 0·03 | 78 |
| | | 0·015 | 98 |
| | Sulphadiazine | 60 | 4·5 |
| | | 30 | 42 |
| | | 15 | 72 |
| Proguanil-pyrimethamine resistant (P.63) | Proguanil | 50 | 92 |
| | Pyrimethamine | 6·0 | 103 |
| | Sulphadiazine | 30 | <1 |

* Parasitized cells in the treated birds shown as a percentage of the parasitized cells in the untreated controls. Hence the lower the percentage the more effective is the particular dose.

TABLE II

CROSS-RESISTANCE TESTS BETWEEN PROGUANIL- AND PYRIMETHAMINE-TREATED STRAINS OF *P. GALLINACEUM*

| Strain | Drug | Dose (mg./kg.) | % Parasitaemia* Passage No. 1 | 3 | 6 | 7 | 9 |
|---|---|---|---|---|---|---|---|
| Proguanil-treated | Proguanil | 50 | | | | | 120 |
| | | 25 | | | | | 86 |
| | | 10 | | | 39 | 93 | |
| | | 4 | | 25 | | | |
| | | 2 | 7·2 | 39 | | | |
| | | 1 | 39 | | | | |
| | Pyrimethamine | 0·12 | | | | | 2·8 |
| | | 0·06 | | 19 | 34 | 57 | 71 |
| | | 0·03 | | 84 | | | 107 |
| Pyrimethamine-treated | Proguanil | 2 | | <2 | 25 | 34 | 6·5 |
| | | 1 | | 28 | | | 26 |
| | Pyrimethamine | 0·06 | 11 | 17 | 35 | 32 | <3 |
| | | 0·03 | 61 | 140 | | | 26 |
| | | 0·015 | 88 | | | | |

* As in Table I.

The proguanil-treated strain soon acquired high resistance, and at the same time slight but definite cross-resistance, to pyrimethamine. At this time, the strain treated under the same conditions with pyrimethamine had not become distinguishable from the parent strain in sensitivity to either drug.

*Potentiation.*—Table III shows the effect of giving both proguanil and pyrimethamine in different proportions to groups of infected chicks.

The ED50's are plotted in Fig. 1. Points lying below the straight line joining the ED50's of the

TABLE III

THE COMBINED ACTION OF PROGUANIL AND PYRIMETHAMINE ON *P. GALLINACEUM*

| Proguanil (mg./kg.) | % Parasitaemia Pyrimethamine (μg./kg.) — | 7·5 | 15 | 30 | 60 | ED50 Pyrimethamine with Appropriate Dose Proguanil† (μg./kg.) |
|---|---|---|---|---|---|---|
| — | 100* | | 65 | 24 | <1·2 | 22 |
| 0·25 | | | 72 | 11 | <1·2 | 20 |
| 0·5 | 81 | 68 | 56 | 7·1 | | 16 |
| 1·0 | 58 | 62 | 20 | <1·2 | | † |
| 2·0 | 11 | | | | | |

* Untreated controls; percentage parasitaemia as in Table I.
† ED50 proguanil alone 1 mg./kg.

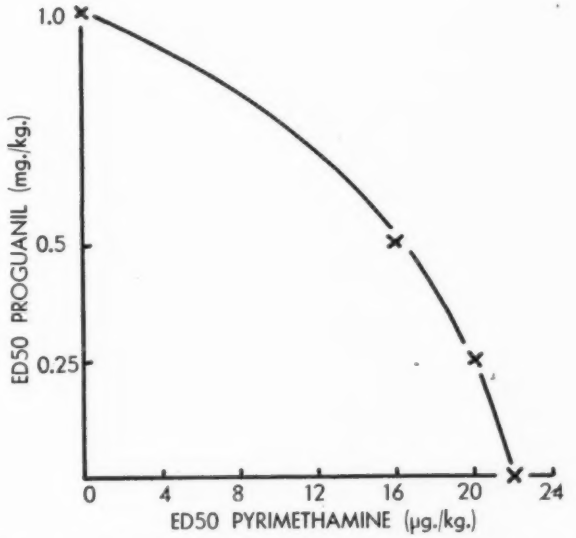



FIG. 1.—ED50's (doses reducing parasitaemia to 50% of the mean parasitaemia of untreated controls) of proguanil and pyrimethamine administered both singly and together in various proportions, in chicks infected with *P. gallinaceum*. ED50's estimated graphically from dose-response curves. For interpretation see text. Note lack of potentiation.

TABLE IV

THE COMBINED ACTION OF SULPHADIAZINE AND PYRIMETHAMINE ON *P. GALLINACEUM*

| Sulphadiazine (mg./kg.) | % Parasitaemia Pyrimethamine (μg./kg.) — | 1·9 | 3·8 | 7·5 | 15 | 30 | 60 | ED50 Pyrimethamine with Appropriate Dose Sulphadiazine (μg./kg.)† |
|---|---|---|---|---|---|---|---|---|
| — | 100* | | | | 84 | 62 | 4 | 34 |
| 0·19 | | | | 116 | 78 | 52 | <1·5 | 26 |
| 0·38 | | | | 82 | 46 | 18 | <1·5 | 14 |
| 0·75 | | | | 79 | 22 | <1·5 | | 11 |
| 3 | | | 57 | 13 | 1·5 | | | 4·1 |
| 6 | 97 | | | | | | | |
| 12 | 91 | 40 | 3 | <1·5 | | | | 1·7 |
| 24 | 57 | | | | | | | |

* Untreated controls; percentage parasitaemia as in Table I.
† ED50 sulphadiazine alone 26 mg./kg.

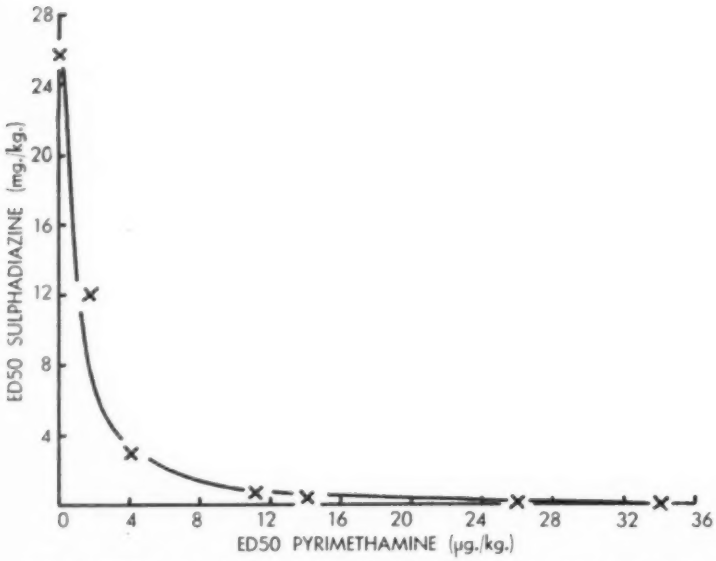



FIG. 2.—ED50's of pyrimethamine and sulphadiazine, administered both singly and together in various proportions, in chicks infected with *P. gallinaceum*. Conventions as in Fig. 1. Note marked potentiation.

two drugs given alone indicate potentiation. The action of pyrimethamine was not potentiated by proguanil (Fig. 1), but was greatly potentiated by sulphadiazine (Table IV, Fig. 2). The point on the curve nearest the origin indicates the optimum combination of doses which will produce the measured effect. Here this proportion is 1/7th of the ED50 of sulphadiazine with 1/8th of the ED50 of pyrimethamine.

The potentiating action of sulphadiazine might possibly be due to an action upon intestinal organisms which normally synthesize metabolites essential to the malarial parasite. The effect of giving poorly absorbed sulphonamides and antibiotics which act upon the intestinal flora is shown in Table V. These results show clearly that the potentiating effect was due, not to the effect in the gut, but to the sulphonamide in the blood. Succinylsulphathiazole was found in the blood only at a very low level and did not potentiate, whereas sulphaguanidine reached a much higher level and did potentiate. The antibiotics had no potentiating effect.

*Inhibition.*—The inhibitory action of PAB and folic acid upon the activity of sulphadiazine and pyrimethamine is shown in Table VI. This inhibition was essentially competitive in nature. The effect of PAB upon pyrimethamine was barely significant ($P=0.1$) because of the large variance of the results. Repetition of the experiment gave similar figures. The degree of antagonism was small, perhaps because pyrimethamine persists in the blood and is therefore difficult to antagonize with a compound which is probably rapidly eliminated. Further work is necessary to investigate this aspect of the problem.

Results obtained using amino-an-fol with sulphadiazine are also included in Table VI ; it acted in the same manner as folic acid. Although chromatographic examination of the sample did not disclose any PAB, there were impurities present and these may have affected the result.

TABLE V

THE EFFECT OF POORLY ABSORBED SULPHONAMIDES AND ANTIBIOTICS ON THE ANTIMALARIAL ACTION OF PYRIMETHAMINE ON *P. GALLINACEUM*

| Pyrimethamine (mg./kg.) | Controls | Sulphaguanidine (10 mg./kg.) | | Succinylsulphathiazole (10 mg./kg.) | | Penicillin 50 ku./kg. | Streptomycin 50 ku./kg. |
|---|---|---|---|---|---|---|---|
| | | Antimalarial Effect | Peak Blood Level (Single Dose) | Antimalarial Effect | Peak Blood Level (Single Dose) | Antimalarial Effect | Antimalarial Effect |
| None | 100* | 102 | 0·28 mg.% at 2 hours† | 100 | Trace† | 77 | 106 |
| 0·03 | 19 | < 1·3 | | 23 | | 36 | 36 |

* Untreated controls; percentage parasitaemia as in Table I.
† A single oral dose of sulphadiazine at 10 mg./kg. gave a peak blood level of 0.84 mg.% at 1 hr.

TABLE VI

COMPETITIVE ANTAGONISM OF SULPHADIAZINE BY PAB AND OF SULPHADIAZINE AND PYRIMETHAMINE BY PGA IN *P. GALLINACEUM*

| Antagonist | | Antimalarial: Sulphadiazine (mg./kg.) | | | | | Pyrimethamine (mg./kg.) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | None | 15 | 30 | 60 | 120 | None | 0·03 | 0·06 |
| PAB (mg./kg.) | None | 100* | 49 | 2·5 | | | 100* | | 6·3 |
| | 0·25 | | | 41 | | | | | |
| | 1·0 | | | | 2·5 | | | | |
| | 4·0 | | | 85 | | | | | |
| | 16·0 | 90 | | | 85 | | | | |
| | 30·0 | | | | | | 103 | | 15·9 |
| Folid acid (mg./kg.) | None | 100* | 76 | < 1·2 | | | 100* | 58 | 2·8 |
| | 10 | | | | | | | 71 | 15 |
| | 25 | | | 98 | 53 | | | | |
| | 50 | | | | 89 | 52 | | | |
| | 100 | 93 | | | | 80 | 97 | 92 | 32 |
| Amino-an-fol (mg./kg.) | None | 100* | 87 | 22 | 6·9 | | | | |
| | 20 | 107 | | 99 | 54 | | | | |

* Untreated controls; percentage parasitaemia as in Table I.
(Each rectangle enclosed in bold rules contains the results of a separate experiment, i.e., this table is made up from 5 experiments.)

## Discussion

*Mode of Action of Sulphonamides.*—It is commonly recognized from studies on bacteria that sulphonamides containing an $NH_2.C_6H_4.SO_2.NH$– group act by competing with PAB. The antimalarial action of sulphonamides may also depend on a similar mechanism because PAB reverses it (Maier and Riley, 1942) and because PAB is important for the metabolism of malarial parasites, particularly *P. berghei*, which is extremely sensitive to sulphadiazine (Hawking, 1953b).

*Resistance and Cross-resistance.*—There are at least four possible explanations of the mechanism of sulphonamide resistance. (1) The resistant organism may produce sufficient PAB to carry on the inhibited metabolic reaction. Thus Landy, Larkum, Oswald, and Streightoff (1943) found that resistant staphylococci produce up to 100 times more PAB than did normal strains. (2) The inhibited reaction may be by-passed by a related action not affected by sulphonamides ; or may be made unnecessary by the direct utilization of the end-product of the reaction (Work and Work, 1948). (3) The resistant strain may develop enzymes for the conversion of sulphanilamide into an essential metabolite, as suggested by the results of Emerson and Cushing (1946) with sulphonamide-resistant neurosporae. (4) The permeability of the cell membrane may be altered so that the sulphonamides are selectively excluded ; or spatial rearrangement of cell receptors may restrict the attachment of the sulphonamide molecule but not that of PAB.

Thurston (1953) put forward the tentative suggestion that sulphadiazine resistance in *P. berghei* may be due to the increased production of PAB. Nevertheless, from recent but still incomplete work, showing that malarial infections—particularly with *P. berghei*—(Hawking, 1953b), do not progress normally in the absence of dietary PAB, it seems unlikely that malarial parasites can synthesize PAB. The experimental conditions are critical and any departure from a strict PAB-deficient diet may give conflicting results (Maegraith, 1954) ; other substances besides PAB may be involved in the suppression of malaria by milk (Maegraith, 1953 ; Refaat and Bray, 1953).

Previous work on cross-resistance between sulphonamides, proguanil and pyrimethamine upon several species of *Plasmodium* (Thurston, 1953), together with the results shown in Table I, lead to the conclusions that

(a) Strains resistant to sulphonamides are usually resistant to proguanil and to pyrimethamine.

(b) Strains resistant to proguanil are usually resistant to pyrimethamine and *vice versa.*

(c) Strains resistant to proguanil and to pyrimethamine are not usually resistant to sulphonamides.

Although some reported results conflict with these conclusions there is usually a reason for the discrepancy. Thus Thurston (1953) and Rollo (1951) reported strains of *P. cynomolgi* and *P. gallinaceum* respectively which, while resistant to proguanil, retained sensitivity to pyrimethamine. On the other hand, Robertson, Davey and Fairley (1952) and Singh, Ray, Basu and Nair (1952) reported strains of *P. gallinaceum* and *P. knowlesi* which became cross-resistant to pyrimethamine after treatment with proguanil. The strains used by Thurston and Rollo were " old " strains which retained a high degree of resistance to proguanil, but which had not been exposed to that drug for some time before testing with pyrimethamine. The strains used by Robertson *et al.* and Singh *et al.*, however, had been exposed to proguanil shortly before the cross-resistance tests were carried out. Proguanil may, therefore, under optimal conditions, give rise to resistance to pyrimethamine as well as to proguanil. Pyrimethamine resistance appears to develop less readily, and is more labile in character, than resistance to proguanil (Table II) ; if the strain is left without treatment, sensitivity to pyrimethamine may return.

Rollo (1951) showed that a strain of *P. berghei*, treated in 9 successive passages with sulphadiazine and rendered 4 times less sensitive to the drug than the parent strain, remained sensitive to pyrimethamine. A similar strain, prepared over a longer period by Thurston (1953), and showing a 100-fold increase in resistance to sulphadiazine, was cross-resistant to pyrimethamine. Rollo's strain, however, was only partially resistant, and it is possible that, with an increase in its resistance to sulphadiazine, cross-resistance to pyrimethamine would have appeared.

Bishop and McConnachie (1948) reported that a proguanil-fast strain of *P. gallinaceum* became resistant to sulphadiazine. All other workers have found that strains made resistant to proguanil have retained their sensitivity to sulphadiazine, and Bishop (1951) was unable to confirm the earlier result. This cross-resistance is not dependent upon the pyrimidine part of the molecule, for Bishop and McConnachie (1950a) showed that a strain of *P. gallinaceum* resistant to sulphanilamide was also highly resistant to proguanil.

*Potentiation and Antagonism.*—Greenberg (1949) showed that the action of proguanil is

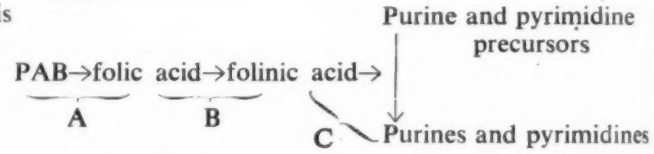
strongly potentiated by sulphadiazine and other PAB competitors which are themselves active against malaria. From the present work it is clear that a similar relationship exists between pyrimethamine and sulphadiazine, but that pyrimethamine and proguanil do not potentiate each other's action.

It is likely that potentiation occurs when two drugs act at different points on the same metabolic pathway, although it is possible that indirect effects—such as the reduction of available metabolite by the action of drugs on the intestinal flora—might produce the same result. It is also likely that an additive effect occurs when the drugs act at the same point or upon different pathways. Therefore, sulphadiazine probably acts at a different point from, but on the same pathway as, pyrimethamine or proguanil. Because of their relationship with sulphadiazine, and because their antimalarial action is antagonized by PAB, it is probable that proguanil and pyrimethamine act at the same point on the same pathway.

The antagonism of sulphadiazine by both PAB and folic acid is competitive and can easily be demonstrated. This has been shown in mice infected with *P. berghei* by Thurston (1954) and in infections of *P. gallinaceum* (see above). Antagonism of proguanil or of pyrimethamine by PAB and folic acid is less effective and less easy to demonstrate. Greenberg (1953), using high doses of PAB and folic acid, has shown in *P. gallinaceum* that proguanil can be competitively antagonized. Thurston (1954), by using a continuous dosing technique, has shown that both proguanil and pyrimethamine can be antagonized by PAB and folic acid in mice infected with *P. berghei*. Thurston's data are too sparse to decide whether the antagonism is competitive or not, except with pyrimethamine and PAB, where competition is observed. Both PAB and folic acid antagonize—the latter competitively—the action of pyrimethamine on *P. gallinaceum* (see above).

Thus in every example in which adequate data have been collected, the compounds have acted as competitive antagonists to all three drugs. The fact that folic acid competitively antagonizes sulphadiazine suggests that folic acid is broken down either by the host or the malarial parasite itself to PAB or *p*-aminobenzoylglutamic acid. The folic acid antagonists amino-an-fol, aminopterin, and amethopterin, are without antimalarial activity (Coatney, Cooper, Eddy and Greenberg, 1953); the first of them has been shown above to antagonize the action of sulphadiazine. Recently, Greenberg (1954) has shown that folic acid, amino-an-fol, and several other folic acid antagonists, competitively antagonize the action of sulphadiazine on *P. gallinaceum*. Plasmodia are probably unable to utilize preformed folic or folinic acid and it is likely that they synthesize these from PAB (Goodwin and Rollo, 1955), which may be supplied by the breakdown of folic acid or of the folic acid antagonists. Greenberg (1954) considers that it is uncertain whether the antagonism of sulphadiazine by substances containing the PAB moiety occurs because the analogues act as sources of folic acid or because the analogues are broken down to PAB which inhibits the sulphonamide.

*Possible Mode of Action of Sulphonamides, Proguanil and Pyrimethamine.*—The biological systems upon which we must assume the action of these antimalarials to take place are shown below.




The facts to be discussed are: (1) strains resistant to proguanil and pyrimethamine retain full sensitivity to sulphonamides, (2) sulphonamide-resistant strains are cross-resistant to proguanil and pyrimethamine, and (3) sulphadiazine potentiates the action of proguanil and pyrimethamine. Although the first and third of these facts suggest that the loci of action of sulphonamides and proguanil-pyrimethamine are not the same, the second suggests a common locus.

The effect of sulphonamides on plasmodia is presumably due to interference with the utilization of PAB in reaction A. The other drugs may, however, act quite differently. The results of cross-resistance tests and the lack of complete antagonism by PAB and folic acid indicate that their action may be more than simple competition with a metabolite. Hitching's evidence (in Goodwin and Rollo, 1954) on the varying ability of folic and folinic acids to reverse the effects of pyrimethamine favours a blockage of reaction B; and if resistance to proguanil and pyrimethamine involved interference with their action at point B only, this would leave reactions A and C vulnerable to other drugs—for example, to the action of sulphonamides on reaction A. So far no drugs are known that interfere with reaction C in plasmodia. Resistance to sulphonamides could be attributed to a by-pass of the whole series of reactions from PAB to folinic acid, thereby render-

ing the parasite insensitive not only to drugs interfering with reaction A but also to drugs interfering at B. Such a by-pass is, however, unlikely because Bishop and McConnachie (1950b) showed that strains treated with sulphadiazine readily became resistant to high doses of proguanil before any sign of sulphonamide resistance became evident. Hitchings (personal communication) has suggested that, in this instance, treatment with sulphadiazine may have resulted in an increase of the efficiency of reaction B. Such an increase in efficiency has been observed in biological systems in response to folic acid antagonists (Broquist, Kohler, Hutchison and Burchenal, 1953). This would account for the early development of proguanil resistance if the action of this drug were upon reaction B. Resistance to sulphonamides would follow as progressively less PAB became necessary to supply reaction B. However, where an increase in the efficiency of reaction B has been observed with bacteria, it has been produced by the direct interference of folic acid antagonists. It seems less likely that such a change would be induced by interference at A.

The experimental findings could be explained by a two-point mode of action for proguanil and pyrimethamine. This would involve, firstly, an acceptor mechanism whereby the drugs are made available to interfere with the metabolic reaction and, secondly, a " lethal " point of action within the metabolic pathway. There may thus be cell receptors responsible for making PAB available to reaction A. Sulphonamides would also be accepted, thus making possible the blocking of reaction A.

Sulphonamide resistance may involve a decrease in cell permeability to the sulphonamide molecule, or a change in distribution or orientation of the receptors, so that, although PAB is still accepted, the sulphonamide molecule is rejected. If pyrimethamine and the active metabolite of proguanil are accepted or rejected by the same mechanism as sulphonamide, sulphonamide-resistance would be accompanied by resistance to these drugs, perhaps before the resistance to sulphonamide became evident.

Resistance to proguanil and pyrimethamine could involve a by-pass of their effect on reaction B, leaving unchanged the sensitivity of the strain to sulphonamide acting at A. On the other hand, a reorientation of PAB receptors, insufficient in extent to cause rejection of sulphonamide molecules, might yet be sufficient to cause rejection of the proguanil-metabolite and pyrimethamine. This would also explain the results in Table II if it were assumed that pyrimethamine has a firmer " foothold " upon the receptors than the proguanil metabolite has. Little change would be needed in the distribution or orientation of the receptors for the complete rejection of the proguanil metabolite, the smallness of the change being reflected in the ease with which resistance can be induced. Pyrimethamine-resistance, on the other hand, may involve a greater change. Further evidence of the effects of small changes of structure in molecules of the pyrimethamine type upon their action against resistant strains is given by Greenberg and Bond (1954) and by unpublished experiments by the author. Some of the phenoxy and benzyl analogues of the pyrimethamine series, when tested against strains of *P. gallinaceum* and *P. cynomolgi* highly resistant to the action of pyrimethamine, showed activity almost as high as against the pyrimethamine-sensitive parent strains of parasite. The analogues, resembling pyrimethamine closely in structure, would be expected to act at the same point in the metabolic reactions; and differences in activity upon resistant strains of parasite can best be accounted for by differences in degrees of acceptance resulting from differences in spatial structure.

From the evidence presented and discussed it is therefore postulated that both pyrimethamine and the metabolite of proguanil enter the metabolic pathway concerned with the uptake and utilization of PAB by the same acceptor mechanism as PAB; the drugs then exert their lethal action on the parasite by interfering with the conversion of folic acid to folinic acid.

## Summary

1. A strain of *P. gallinaceum* resistant to both proguanil and pyrimethamine retained its sensitivity to sulphadiazine.

2. *P. gallinaceum* treated with proguanil quickly became resistant to that drug and slightly resistant to pyrimethamine. Identical treatment with pyrimethamine failed to induce resistance either to pyrimethamine or to proguanil.

3. Sulphadiazine strongly potentiated the action of pyrimethamine. There was no potentiation between proguanil and pyrimethamine.

4. The action of both sulphadiazine and pyrimethamine was competitively antagonized by folic acid.

5. A possible mechanism of action depicting the interrelationship of sulphadiazine, proguanil and pyrimethamine is put forward. Proguanil and pyrimethamine may have a twofold mode of

action involving, firstly, an acceptor mechanism whereby the drugs are made available to interfere with the metabolic reaction and, secondly, a " lethal " point of action within the metabolic pathway—probably by interfering with the conversion of folic to folinic acid.

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# THE RELEASE OF HISTAMINE BY POLYMYXIN B AND POLYMYXIN E

BY

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Some of the side-effects of polymyxins B and E in man and experimental animals might be due to the liberation of histamine by these antibiotics. Such a liberation could account for the "triple response" on injection of the histamine-free antibiotics in man (Swift and Bushby, 1953), and the close similarity in the dog between the depressor action of massive doses of polymyxins B and E (Brownlee, Bushby, and Short, 1952) and that of the histamine liberator 48/80 (Baltzly, Buck, de Beer, and Webb, 1949; Paton, 1951). It would also provide a rational explanation for the subjective side-effects of polymyxin B in man being relieved by antihistamine drugs (Kagan, Krevsky, Milzer, and Locke, 1951). Evidence that the polymyxins do in fact release histamine in the dog and the rat and have other properties in common with known histamine liberators is presented here.

## METHODS

The polymyxins used in these experiments were therapeutic samples prepared for parenteral injection and were free from histamine (less than 0.1 μg. histamine/mg.). The quantities are expressed in mg. on the basis that pure polymyxin B and E contain 10.000 u./mg. The polymyxin E had a potency of 8,000 u./mg. and the polymyxin B 7,000 u./mg.

*Rats.*—Rats weighing 80–150 g. from an inbred Wistar colony were used in all experiments. To determine effects on skin histamine, rats were killed by a blow on the head at various intervals after dorsal subcutaneous injection of the polymyxins or 48/80; areas of skin of approximately 2 cm.$^2$ were removed from the ventral abdomen and transferred to trichloracetic acid as rapidly as possible. The histamine was extracted in the manner used by Haas (1940) and assayed on isolated guinea-pig ileum. To assess oedema formation, groups of five rats were given water by stomach tube (5% of the body-weight) immediately before subcutaneous injection of polymyxin or 48/80 and the diameters of the feet measured with micrometer callipers. When antihistamines were used, they were injected subcutaneously 10 min. before polymyxin or 48/80.

*Intradermal Effects.*—The increase in capillary permeability around the site of the intradermal injection of polymyxin B, E, or 48/80 (in 0.1 ml.), in groups of at least six guinea-pigs of 350–400 g., was determined by the procedure described by Miles and Miles (1952), except that the Evans Blue (0.5 ml. of a 1% soln.) was injected directly into the heart instead of intravenously. The area of blueness on the external surface of the skin was measured.

*Dogs.*—The effect of intravenous polymyxin or 48/80 on the carotid blood pressure, the packed cell volume, and the histamine content of heparinized blood (or plasma) drawn from the femoral artery, were determined in dogs anaesthetized with pentobarbitone sodium. Packed red cell volumes were measured after centrifuging at 1,500 *G.* for 30 min. in a haematocrit. The histamine equivalent of the plasma itself or of extracts of whole blood was determined on the carotid blood pressure of cats under pentobarbitone sodium, or on isolated guinea-pig ileum, or both. The method of Code (1937) was used to extract histamine from blood and was adapted for extraction of skin as by Haas (1940). The skin histamine concentration after polymyxin was compared with that of similar pieces of skin from the opposite flank removed before giving the polymyxin.

*Cats.*—The depressor action of the polymyxins and 48/80 in cats was also determined under pentobarbitone anaesthesia.

## RESULTS

### *Effects in Rats*

Polymyxins B and E and also 48/80 caused vasodilatation, and oedema of the muzzle and limbs in the rat. These effects were associated with the depletion of skin histamine.

Comparison of the histamine concentrations of the skin taken remotely from the injection site at various intervals after subcutaneous injection has shown that polymyxin B is a more powerful histamine liberator in the rat than is 48/80 (Fig. 1). After 5 mg./kg. of either substance the skin histamine was reduced by about 80% in four hours and did not return to normal levels until about 10

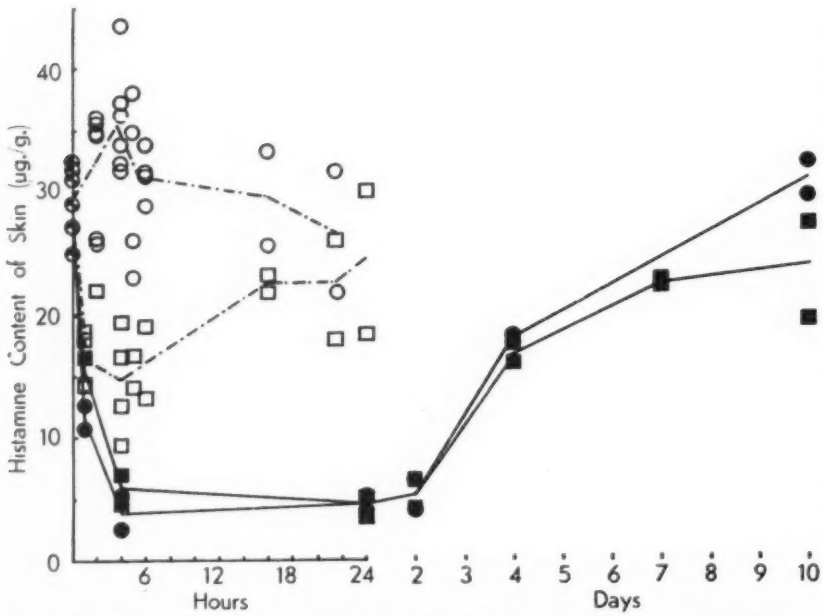


FIG. 1.—The histamine content of the skin of rats at intervals after the subcutaneous injection of polymyxin B or 48/80. Open squares, polymyxin B, 1 mg./kg.; filled squares, polymyxin B, 5 mg./kg.; open circles, 48/80, 1 mg./kg.; filled circles, 48/80, 5 mg./kg.; half-filled circles, controls.

days later. Whereas 1 mg./kg. polymyxin B temporarily reduced the skin histamine by about 50%, the same dose of 48/80 appeared to increase the histamine content of the skin—extracts of the skin removed 4 hr. after 1 mg./kg. 48/80 contained significantly more histamine than the controls ($P=0.01$–$0.02$). (An increase followed by a decrease in extractable skin histamine after local application of mustard oil in rats has been described by Haas (1940).) Polymyxin E also depleted the skin histamine, but was not so active as polymyxin B or 48/80. The skin histamine concentration 24 hr. after 5 mg./kg. polymyxin E was about 30% below normal (3 rats), and at 4 and 24 hr. after 10 mg./kg. polymyxin E it was reduced by 74% (2 rats) and 58% (3 rats) respectively. Although it is a more powerful histamine liberator, polymyxin B (LD50 approx. 50 mg./kg.) is no more toxic than 48/80 when given subcutaneously to rats.

Fawcett (1954) found that the intraperitoneal injection of 48/80 in a large volume of Tyrode caused a release of histamine into the fluid in the peritoneum and, as previously described by Mota, Beraldo, and Junqueira (1953), a loss of granules from the mast cells of the mesentery. When injected in the same way, 5 mg./kg. polymyxin B in 20 ml. affected the mast cells in the same manner as did 5 mg./kg. 48/80; in two rats given polymyxin the histamine content of the peritoneal fluid reached higher concentrations (3.3 and 3.4 µg./ml.) than it did in two others given 48/80 (1.7 and 2.0 µg./ml.). Loss of granules from mast cells of the mesentery also occurred after subcutaneous injection of 5 mg./kg. of either polymyxin or 48/80. Norton and de Beer (1955) have shown that polymyxin B causes fragmentation of rat mast cells *in vitro*.

Oedema in hydrated rats caused by either 5 mg./kg. polymyxin B or 10 mg./kg. 48/80 was not reduced even by massive doses of antihistamines (chlorcyclizine 100 mg./kg. or α-1-(4-methylphenyl) - 1 - (2 - pyridyl) - 3 - pyrrolidino prop-1-ene monohydrochloride [" Actidil "] 20 mg./kg.).

*Intradermal Response*

Swift and Bushby (1953) noted that the intradermal injection of polymyxin E produced a typical histamine " triple response." In guinea-pigs injected with Evans Blue, polymyxin B or E caused a leakage of the dye similar to that produced by somewhat smaller concentrations of 48/80. The mean size of lesions with 0.5, 2, and 8 µg. polymyxin were 2.1, 4.7, and 7.2 mm. respectively, and with 0.25, 1, and 2 µg. 48/80 were 3.5, 5.3, and 7.3 mm. respectively; the mean value for saline was 2.8 mm.

*Effects in Dogs*

*General.*—The fall in blood pressure caused by large intravenous doses of polymyxin B or E in anaesthetized dogs is delayed, and resembles that with 48/80. With 19 injections of polymyxin E the average delay was $13.9 \pm 4.1$ sec., and with 8 injections of 48/80 the average delay was $10.9 \pm 4.3$ sec. A typical fall in a dog given 5 mg./kg. polymyxin B is illustrated in Fig. 2. Such falls are associated with a rise in the concentration of circulating histamine, but the concentrations reached are not as high as after 48/80. Histamine was estimated either by testing the plasma itself or by testing an extract of whole blood prepared by Code's method. Both the untreated plasma and the extracts of blood obtained shortly after inject-

ing large doses of polymyxin B or E had a depressor action in the anaesthetized cat and a spasmogenic action on isolated guinea-pig ileum greater than that of samples taken before injection of polymyxin. Parallel quantitative assays with histamine showed fair agreement. The samples were as sensitive to the antihistamine "Actidil," and as resistant to atropine, as was histamine, although a slight effect of the plasma samples persisted after the antihistamine was given.

The rise in circulating histamine was associated with a slight fall in the skin histamine of two dogs. In the experiment illustrated in Fig. 2 the skin histamine fell from 8.6 μg./g. to 3.5 μg./g. 2 hr. after injection of the polymyxin.

*Comparison with 48/80.*—Polymyxins B and E are much less powerful histamine-liberators in the dog than is 48/80, and polymyxin B seemed to be slightly less active than polymyxin E.

Polymyxin B at 5 mg./kg. lowered the blood pressure by 85–135 mm. Hg in each of four dogs. The concentration of circulating histamine did not increase sufficiently in two of these animals for its estimation by direct testing of the plasma itself, but in the two other dogs extracts of the blood showed a small increase in circulating histamine after injection of the polymyxin B (Fig. 2). In another dog 10 mg./kg. polymyxin B raised the concentration of plasma histamine from less than 0.05 μg./ml. to 0.1–0.15 μg./ml. within 1 min., the plasma histamine falling to 0.08 μg./ml. within 4 min. and to below 0.05 μg./ml. within 10 min. Polymyxin E at 5 mg./kg. lowered the blood pressure by 90–130 mm. Hg in 9 out of 10 dogs, one animal being refractory. The plasma histamine concentration generally rose within 1 min. to between 0.1 and 0.3 μg./ml., and then declined rapidly. The packed red cell volume increased from initial values of 38–41% to 39–49% in 3 min. and to 40–52% in 10 min. In two dogs, 2 mg./kg. polymyxin E caused depressions of 70 and 120 mm. Hg respectively and increased the plasma histamine from less than 0.05 μg./ml. to approximately 0.1 μg./ml.

Both the duration of the depressor action of polymyxin and the increase in plasma histamine are small compared with that caused by 48/80. This at 0.2 mg./kg. commonly increases the plasma histamine to 1 μg./ml. or more. Confirmation that polymyxin E is considerably less than one-tenth as active as 48/80 was obtained by injecting both compounds in the same dog. Polymyxin E, 2 mg./kg., produced a fall from 166 to 46 mm. Hg and 6 min. later the blood pressure was 116 mm. Hg; the highest plasma histamine concentration found was 0.1 μg./ml. One hr. later, when the blood pressure had returned to 146 mm. Hg, 0.2 mg./kg. 48/80 lowered the blood pressure to 44 mm. Hg, and after 6 min. the blood pressure was only 60 mm. Hg: the plasma histamine reached a value of 1 μg./ml.

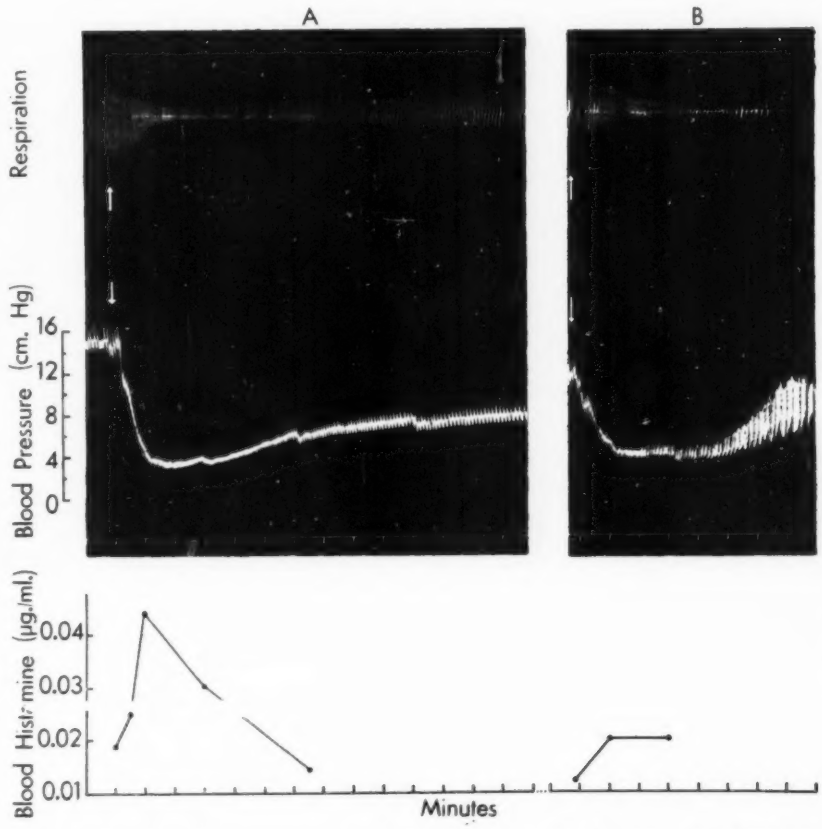


FIG. 2.—The effect of intravenous polymyxin B on respiration, blood pressure, and blood histamine concn. in an anaesthetized dog. A, a first dose of 5 mg./kg. polymyxin B; B, a similar dose 2½ hr. later. Time 1 min.

*Tachyphylaxis.*—Brownlee *et al.* (1952) noted tachyphylaxis to the depressor action of polymyxin. We have found that the tachyphylaxis is associated with a smaller

liberation of histamine with the second than with the first injection. This is illustrated for polymyxin B in Fig. 2. In another animal 5 mg./kg. polymyxin E lowered the blood pressure by 122 mm. Hg and produced a plasma histamine level of 0.2 μg./ml.; one hr. later a second dose of 5 mg./kg. caused a fall of similar magnitude but of much shorter duration, and the plasma histamine did not exceed 0.05 μg./ml.

*Other Effects.*—The very large doses of polymyxins B and E (5 and 10 mg./kg.) used in the foregoing experiments approximate to lethal doses. The toxic effects consist of severe vascular engorgement, particularly of the liver, and a resulting fall in blood pressure and respiratory depression. Following intravenous polymyxin B or E, after brief apnoea, the breathing was often temporarily increased in rate and depth, but after 1 to 2 min. the tidal volume nearly always decreased, and then breathing became less frequent and sometimes stopped. At this stage most but not all dogs could be saved by artificial respiration. The effects of polymyxin on respiration are cumulative, even when a period of 2½ hr. elapsed between doses (Fig. 1) and this contrasts with the tachyphylaxis to the depressor effect. Bronchoconstriction is probably largely responsible for the decrease in tidal volume, and was shown in one deeply anaesthetized dog by the method of Konzett and Rössler, (1940).

Prior administration of massive doses of "Actidil" (10 mg./kg.) intravenously to each of four dogs reduced the rate of the fall in blood pressure caused by 5 mg./kg. polymyxin B or E, without significantly altering its ultimate extent or decreasing the toxic respiratory effects of the polymyxin.

*Effects in Cats*

Given intravenously to cats polymyxin B and E caused a delayed depressor response resembling that with 48/80. The effect was negligible (less than 20 mm. Hg) with 2 mg./kg. polymyxin B in 6 of 7 cats and in one amounted to only 26 mm. Hg; depressions of 30–85 mm. Hg were caused in 4 cats given 5 mg./kg. polymyxin B. Since as little as 0.1 mg./kg. 48/80 causes a profound fall in blood pressure (Paton, 1951) polymyxin B appears to be a much less powerful histamine liberator than 48/80 in the cat.

Bronchial tone, measured by the method of Konzett and Rössler (1940) in a cat under deep chloralose anaesthesia, was slightly increased by 5 mg./kg. polymyxin B and very greatly increased for over an hour by 12 mg./kg.

## Discussion

The polymyxins B and E release tissue histamine, their potency in this respect varying relative to that of 48/80 under different conditions. Subcutaneously in rats they are about as active as 48/80 in releasing histamine from skin remote from the site of injection, and in causing degranulation in the mast cells of the mesentery. Intravenously in dogs their action is feeble, but the histamine liberated is sufficient to account for at least part of their delayed depressor action. In these animals the rise in circulating histamine and the skin depletion are small compared with those caused by 48/80, even when the blood pressure falls to very low levels. As these investigations were made on material containing 7,000–8,000 u./mg. the action may be due to an impurity; this is unlikely, however, because Brownlee *et al.* (1952) found that the depressor action was produced by even the purest material (10,000 u./mg.).

Although antihistamines do not abolish the effects of the polymyxin, the rate of fall in blood pressure caused by polymyxin B or E was slower after an antihistamine, which suggests that the effects are at least modified. The lessening of some of the subjective side-effects by the concomitant use of antihistamines reported by Kagan *et al.* (1951), together with our finding that polymyxins liberate histamine in other species, suggests that some of the side-effects of polymyxin in man may be due to a slight histamine release.

## Summary

1. Polymyxin B and polymyxin E injected subcutaneously in rats are about as active as 48/80 in releasing skin histamine remote from the site of injection and causing degranulation of the mast cells in the mesentery.

2. In contrast, when injected intravenously in dogs and cats, their histamine-releasing activity is not powerful.

3. In dogs they cause a small rise in circulating histamine, and this is associated with their depressor and bronchoconstrictor effects in this species.

4. These observations may be related to the finding that some of the subjective side-effects of polymyxin in man are relieved by antihistamine drugs.

The authors are indebted for technical assistance to Mr. A. Boura.

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# PHASE-BOUNDARY POTENTIALS AND GANGLION-STIMULANT ACTIVITY

BY

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The theory that nerve action potentials are phase-boundary potentials evoked by acetylcholine in contact with nerve lipoid has been advocated in recent years by Barnes and Beutner (1946, 1947, 1949a, 1949b, 1949c). It is based on the demonstration (Beutner and Barnes, 1941) that weak solutions of acetylcholine develop negative potentials in contact with certain oily substances under the artificial conditions of an "oil cell," such as is shown diagrammatically in Fig. 1. Feldberg (1945) considered the possibility that such boundary potentials might be associated with the depolarizing property of acetylcholine at autonomic ganglia and at motor endplates.

Furthermore, after measuring phase-boundary potentials of several other substances, Barnes and Beutner (1949c) concluded that the different actions produced by altering the chemical structure of acetylcholine could be explained in terms of phase-boundary potentials.

It seemed to us that the choline esters and ethers previously examined by Hey (1952) would be very suitable drugs with which to test the validity of the Barnes-Beutner theory; for, if it is tenable, then the phase-boundary potentials developed by drugs highly active as nerve-cell stimulants should be high, whereas those developed by drugs relatively weak in this respect should be low.

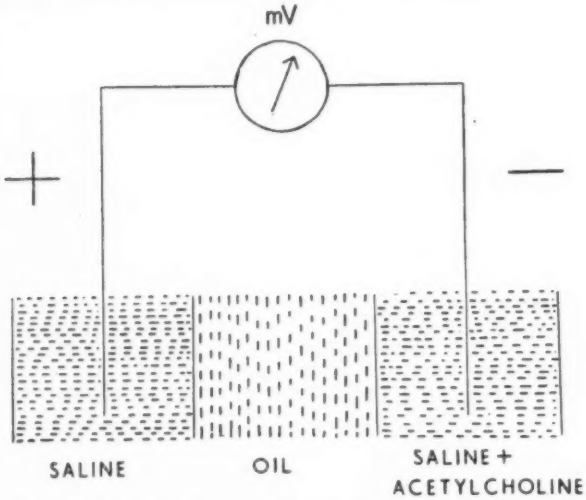


FIG. 1.—Schematic diagram of an "oil cell" for demonstrating phase-boundary potential developed at the interface between solution of acetylcholine and an oil phase.

## METHODS

We used oil cells similar to those described by Beutner and Barnes (1941) and Barnes and Beutner (1942). Re-distilled guaiacol was used for the oil phase, since these authors claimed (1947) that guaiacol has certain physical properties in common with protoplasm—for example, its electrical conductivity, and its ability to absorb lipoid-soluble substances.

Solutions of 0.003 M of each of the esters and ethers were made up in 0.7% saline and tested in the modified oil cell shown in Fig. 2. This consisted of a glass U-tube provided with a drain tap to allow thorough cleaning after each separate estimation. 2 ml.

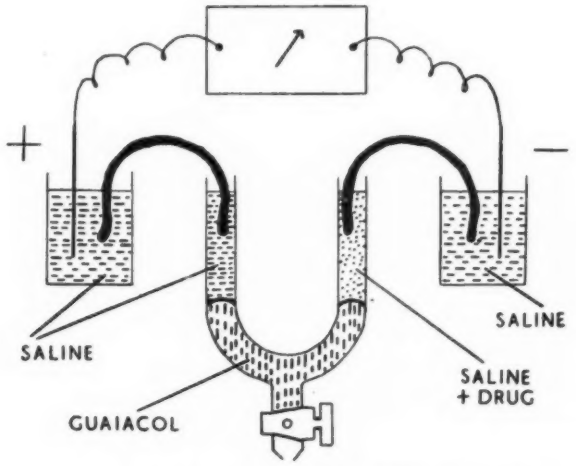


FIG. 2.—Diagram of apparatus used in this investigation of phase-boundary potentials developed by solutions of esters and ethers of choline in contact with guaiacol. Electrical continuity between the limbs of the U-tube and the outer saline-containing vessels is achieved by saline wicks; the measuring instrument is connected to chlorided silver wire electrodes dipping into the two outer vessels.

guaiacol was first run into the tube, followed by 1 ml. saline into the left-hand limb and 1 ml. drug solution into the right-hand limb. Each limb was connected, by a saline wick, to an outer vessel containing 0.7% saline. Chlorided silver wire electrodes dipped into the outer vessels, and the potential between these was measured on a Cambridge *p*H meter set to read millivolts. Three estimations were made for each drug solution and the mean potential calculated.

A few experiments were performed using lecithin instead of guaiacol; membranes consisting of frog's skin (Barnes, 1940) were also tried.

## RESULTS

### *Choline Esters and Nicotine*

The phase-boundary potentials developed between 0.003 M solutions of the esters and guaiacol, and between nicotine acid tartrate and guaiacol, are shown in Table I; in the second column are the means of several determinations for each drug

TABLE I

PHASE-BOUNDARY POTENTIALS AND GANGLION-STIMULANT ACTIVITIES OF CHOLINE ESTERS AND NICOTINE

Except for methacholine, relative stimulant activities are from data of Hey (1952)

| Compound | Mean Potential (mV) Developed by 0·003 M Solutions Against Guaiacol | Molar Ganglion-stimulant Activity, Relative to Choline Phenyl Ether = 100·0 |
|---|---|---|
| Acetylcholine .. .. | 35 | 2·2 |
| Methacholine .. .. | 45 | 0 |
| Carbachol .. .. | 18 | 7·0 |
| Propionylcholine .. | 47 | 3·4 |
| *iso*Butyrylcholine .. | 50 | 8·4 |
| Trimethylacetylcholine .. | 51 | 21·4 |
| Benzoylcholine .. .. | 68 | 2·5 |
| Phenacetylcholine .. | 70 | 7·4 |
| Nicotine acid tartrate .. | 41 | 47·5 |

—seven for acetylcholine, and three for each of the other drugs—expressed to the nearest mV. The value for acetylcholine, incidentally, agrees closely with that quoted by Barnes and Beutner (1949c). Data in the third column indicate the corresponding molar ganglion-stimulant activities relative to choline phenyl ether = 100.0, as previously determined by Hey (1952) upon the vasomotor ganglia of cats.

As will be seen from Table I, there seems to be little correlation between ganglion-stimulant activity and phase-boundary potentials within this series. Methacholine, though not specifically tested by Hey, is well known to be devoid of nicotinic activity: nevertheless it develops a potential of 45 mV. Carbachol, on the other hand, gives a relatively low potential of 18 mV. Larger ester groupings, as in benzoylcholine, tend to develop high potentials without, necessarily, having enhanced nicotinic activity. Conversely, the high stimulant activity of nicotine acid tartrate is not associated with a high boundary potential.

### *Choline Ethers*

In Table II the choline ethers tested are arranged in descending order of ganglion-stimulant activity. It will be noted that members of this series exhibit a wide range of stimulant activity (370 to 0.4) accompanied by a minimum of structural alteration. This wide variation in pharmacological activity is not, however, reflected in the phase-boundary potentials; these are consistently high throughout the series and are probably dominated by the affinity of the aryl moieties for the oil phase.

TABLE II

PHASE-BOUNDARY POTENTIALS AND GANGLION-STIMULANT ACTIVITIES OF CHOLINE ETHERS

| Compound $R\text{–}O.CH_2.CH_2.N(CH_3)_3Br$ R = | Mean Potential (mV) Developed by 0·003 M Solutions Against Guaiacol | Molar Ganglion-stimulant Activity, Relative to Choline Phenyl Ether = 100·0 |
|---|---|---|
| *m*-Bromophenyl .. .. | 75 | 370 |
| 3: 5-Dibromophenyl .. | 69 | 337 |
| *m*-Chlorophenyl .. .. | 76 | 220 |
| Phenyl .. .. .. | 76 | 100 |
| *m*-Tolyl .. .. .. | 78 | 13·1 |
| *p*-Chlorophenyl .. .. | 75 | 10·4 |
| 3: 5-Xylyl .. .. | 75 | 5·2 |
| *p*-Tolyl .. .. .. | 79 | 0·4 |

### *Effect of Time and Concentration on Potentials*

The phase-boundary potentials developed by the choline ethers (Table II) were measured immediately after the drug solutions reached the guaiacol. If the drug solutions were allowed to remain in contact with the guaiacol phase for 10 min. before the potential was measured, slightly lower readings were obtained. However, the proportionate reduction in voltage was the same for each drug.

We have also investigated the effect of altering the molar concentration of the ether solutions and have been able to confirm the observation of Beutner and Barnes (1941) that the size of the phase-boundary potential developed by a solution of a choline derivative is proportionate to the logarithm of its concentration.

### *Tests on Other Boundary Systems*

In an attempt to imitate physiological conditions more closely, certain of the ethers were tested on cell models in which lecithin replaced guaiacol, or in which a partition of frog's skin was used. The

potentials developed, though always less than one tenth of those obtained with guaiacol, showed a similar consistency throughout the ether series. Thus, a closer approach to "physiological" conditions afforded no closer correlation between phase-boundary potentials and stimulant activity.

## Discussion

On the basis of their theory of phase-boundary potential, Barnes and Beutner (1949b) have boldly rejected some of those present-day views of drug action which embody steric considerations; they have challenged Ing's concept of precise receptor fit, and have accused both Ing and Pfeiffer of showing "preoccupation with skeletal rather than dynamic aspects of autonomic drug action." We believe, however, that Barnes and Beutner are themselves preoccupied with phase-boundary potentials to the exclusion of many other important factors.

Not only do many of the observations given here fail to accord with the theory, but certain of those reported by Barnes and Beutner themselves also, in our opinion, fail to do so. For example, the values which they give (Barnes and Beutner, 1949b) for the phase-boundary potentials of equimolar solutions of tetramethylammonium and tetraethylammonium bromides (20 and 74 mV, respectively) do not seem to predict the likely stimulant or blocking actions of these drugs on ganglia or on motor endplates; tetraethylammonium, which blocks sympathetic ganglia without depolarization (Paton and Perry, 1953), would be expected by the theory to evoke a *lower* boundary potential than its stimulant tetramethyl analogue.

Our results with the esters and ethers of choline make it clear that, in measuring phase-boundary potential, we are dealing with an isolated physicochemical property, dependent upon solubility in oil, and that this property has little bearing on the stimulant behaviour of the drugs *in vivo*.

Barnes and Beutner (1949a) claim that the oil-cell method provides a basis for distinguishing between "sympathetic" and "parasympathetic" drugs. (They presumably mean drugs acting upon structures with postganglionic sympathetic and parasympathetic innervations, respectively.) They showed that the "sympathetic" drugs dissolve in triglyceride oils, on which they form negative phase-boundary potentials. In contrast, "parasympathetic" drugs, like acetylcholine, develop boundary potentials with guaiacol but not with triglyceride oils. On the grounds that they have demonstrated a boundary-potential effect between nicotine and tributyrin, they go so far as to imply that the pressor effect of nicotine is a direct adrenaline-like action. Few would accept this suggestion. In any event, we find that nicotine does, in fact, develop a boundary potential of 41 mV in contact with guaiacol.

In conclusion, we agree with Perry (1953) that Barnes and Beutner's models can only be regarded as a vast over-simplification of a most complex situation. The behaviour of drugs on nervous structures must be intimately bound up with other factors besides phase-boundary potential—with molecular size and shape, electron density, lipoid solubility, and probably many other properties.

## Summary

1. Simple apparatus is described for the measurement of phase-boundary potentials developed between solutions of drugs and guaiacol.

2. Examination of the phase-boundary potentials of esters and ethers of choline, and of nicotine, has failed to reveal any obvious correlation with ganglion-stimulant activity.

3. The Barnes-Beutner "theory of phase-boundary potential" is inadequate to account for the pharmacological behaviour of choline compounds on autonomic ganglia.

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# ACTION OF THE HISTAMINE-RELEASER POLYVINYL-PYRROLIDONE ON CAPILLARY PERMEABILITY IN DOGS

BY

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It is now well established that besides the antigen-antibody complex various substances may produce pathological symptoms in man and animals, very similar to allergic reactions, and attributable to the same mechanism—the liberation of endogenous histamine.

During the last few years we have studied extensively two such substances—polyvinylpyrrolidone (PVP) (Halpern, 1952 ; Halpern and Briot, 1953a, b) and dextran (Briot and Halpern, 1952 ; Halpern and Briot, 1954a and b). Both are polymers of relatively high molecular weight and both are able to release histamine from cells (Briot and Halpern, 1952 ; Halpern and Briot, 1952a and b ; Halpern and Briot, 1954a and b).

It should be emphasized from the beginning that the characteristic symptoms produced by each of these polymers are limited to one particular animal species, for which their action is specific. Thus dextran elicits the characteristic symptoms only in rats, whereas PVP determines an anaphylactoid syndrome only in dogs (Halpern, 1952).

The symptoms produced by the long-chain molecules are similar to those observed in anaphylactic shock and in various clinical allergic conditions. The fundamental change is an alteration in capillary permeability, conditioned by the liberation of endogenous angio-toxic substances. The protective action of adrenaline and of the antihistaminics on these syndromes is, no doubt, related to their well-known effects on capillary permeability (Halpern, 1942 ; Halpern and Cruchaud, 1947 ; Halpern, Guillaumat, and Cruchaud, 1948 ; Briot and Halpern, 1952).

The role of capillary permeability in determining allergic conditions has been emphasized by many authors ; on the other hand much experimental evidence has been provided that the antihistaminics inhibit allergic reaction by altering the functional state of the capillary barrier (Halpern, 1948). For these reasons it seemed worth while to study the modifications in capillary permeability produced by the administration of the histamine liberator PVP in dogs.

## METHODS

The experiments were carried out on adult dogs weighing between 15 and 25 kg. Most of the experiments were done on unanaesthetized dogs ; in some, however, the animals received chloralose as a general anaesthetic.

To measure capillary permeability we used the technique described by Landis, Jonas, Angevine, and Erb (1932), with some minor modifications to adjust to animals the technique used in man.

Dogs were trained to lie untied on their backs on a heated operating table. After 10 min. of rest, a rubber cuff (40 to 80 mm.) wrapped in an inextensible fabric, 50 cm. in length, was rolled at the origin of the front leg. The cuff, connected with a Hg manometer, was inflated to a suitable pressure maintained usually for 30 min. The blood samples, 1 ml. each, were obtained by puncturing superficial veins of the leg. We used a special trocar provided with a stilette, which was left in the vein till the end of the experiment.

Blood samples were taken before any compression or injection of PVP and then 10, 20 and 30 min. afterwards. The blood was collected into dry, clean glass tubes containing 0.1 ml. heparin ("Liquemine," Roche). An aliquot quantity of heparinized blood was poured into Wintrobe tubes and centrifuged for 20 min. at 3,000 rev./min. to determine the haematocrit value.

The plasma proteins were determined by two techniques : by refractometry and by dry weight. For reasons of convenience we usually preferred the refractometric method, having checked its accuracy by weight.

The changes in the haematocrit values, and in the concentration of plasma proteins before and after compression, made possible the calculation of the leakage of water and of proteins through the capillary barrier.

To calculate the leakage of water and of proteins, Landis compared the composition of the blood in the normal arm with the composition of the blood drawn

at the same moment from the veins of the arm which had been subjected to compression of 85 mm. Hg for 30 min.

The leakage of water is calculated according to the formula :

$$X = 100 - 100 \frac{H1}{H2}$$

where X = leakage of water in ml./100 ml. blood, H1 = haematocrit value in the non-compressed arm, and H2 = haematocrit value in the arm compressed for 30 min. at 80 mm. Hg.

Having calculated the loss of water, it is possible to determine the filtration of proteins per 100 ml. of blood according to the formula :

$$\Delta \text{ Pr} = Pr^1 \frac{(pl^1)}{100} - Pr^2 \frac{(pl^1 - X)}{100}$$

where $\Delta$ Pr = leakage of proteins per 100 ml. blood, $Pr^1$ = level of plasma proteins at time 0 (non-compressed arm), $Pr^2$ = level of plasma proteins after 30 min. compression, $pl^1$ = plasma volume of normal blood, and X = leakage of water in ml./100 ml. blood.

Having established the leakage of water (X) and proteins (Pr) per 100 ml. of initial blood, it is easy to calculate the level of proteins in the capillary filtrate (Cf) according to the formula :

$$Cf = \frac{100 \text{ Pr}}{X}$$

PVP was injected intravenously in a dose of 0.25 ml./kg. of a 25% soln. Promethazine (10 mg./kg.) was administered intramuscularly 20 min. before the compression, or injection of PVP. The compression was applied to all animals, with the exception of those which received PVP, at a uniform pressure of 80 mm. Hg, which is about half the mean arterial pressure in our dogs. In dogs treated with PVP, which often produces a severe hypotension, the rate of the compression was variable and adjusted to the fluctuations of the blood pressure in order to maintain a compression equal to half the arterial pressure. The blood pressure was measured with a mercury manometer connected to the femoral artery. Chloralose (100 mg./kg.) was administered intravenously.

## Results

### *Gradient of Capillary Permeability to Water and Proteins Following Compression*

*Unanaesthetized Animals.*—In Table I, group 1, are summarized the mean figures concerning the modifications of the haematocrit values and of the capillary permeability to water and to proteins observed in five dogs when a 30 min. compression at 80 mm. Hg was exerted at the origin of the anterior leg. The filtration rates of water and of proteins have been calculated on the basis of the previously indicated formulae.

Fig. 1 (division A) illustrates the mean values of the water and protein loss during the compression.

The figures indicate that the quantity of water which passes through the capillary wall in 30 min. is an average of 7.9 ml./100 ml. blood (range 4.9 and 12.5 ml.).

As for the proteins which are leaving the circulatory bed following the compression, their amount averages 0.170 g./100 ml. blood (range 0.04 and 0.27 g.). The protein concentration in the capillary filtrate averages 2.10 g./100 ml. (range 0.6 to 3.8) for a compression of 80 mm. Hg.

The analysis of the gradient of this phenomenon shows that the filtration rapidly reaches a constant level, which subsequently does not change very much. Recently, Cachera and Darnis (1951) obtained similar results in man in their clinical research on the permeability of capillaries in hepatic diseases.

*Animals Anaesthetized with Chloralose.*—The effect of anaesthesia was studied on four dogs. The mean results are summarized in Table I, group 2, and represented in Fig. 2 (division A). Apparently, anaesthesia does not alter significantly the filtration of water in normal dogs, while the protein content of the capillary filtrate was higher than normal.

### *Action of PVP on Capillary Permeability in Dogs*

In previous researches (Halpern, 1952 ; Halpern and Briot, 1953a and b) intravenous administration of PVP to dogs produced the following syndrome: 40 to 120 sec. after the injection the animal showed signs of generalized and severe pruritus. The whole skin became wine red, while the arterial blood pressure declined rapidly and stabilized itself between 20 to 60 mm. Hg for 30 to 180 min. The erythema and pruritus subsided in about 30 to 60 min. The animals then often manifested symptoms of shock, and a slight oedema of the eyelids, lips, snout, and of the foot pads was noticeable. In some animals the erythema and pruritus were masked by symptoms of severe shock with prostration, hypothermia, vomiting and bloody diarrhoea.

It was shown by Halpern and Briot (1953a,b) that the common basic feature of all these symptoms was a sudden alteration of capillary permeability. The intense erythema, vasodilatation and haemoconcentration, the extravascular leakage of high molecular dyes and of proteins provided consistent evidence for this vascular disturbance. These authors finally showed that PVP determined in dogs a sudden and massive liberation of endogenous histamine, which could account for the characteristic symptoms (1953b).

TABLE I

HAEMATOCRIT VALUES, CAPILLARY WATER AND PROTEIN FILTRATION RATES, AND CAPILLARY FILTRATE PROTEIN CONTENT IN CONTROL DOGS AND IN DOGS TREATED WITH POLYVINYLPYRROLIDONE

The numerals are means of 4 or 5 determinations, ±S.D. For further explanation see text.

| Group | Time in Minutes: | | 0 | 10 | 20 | 30 | 0 | 10 | 20 | 30 | 10 | 20 | 30 | 10 | 20 | 30 | 10 | 20 | 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Treatment | No. of Dogs | Cell Volume Haematocrit | | | | Plasma Protein (g./100 ml.) | | | | Fluid Loss (ml./initial 100 ml.) | | | Protein Loss (g./initial 100 ml.) | | | Protein in Filtrate (g./100 ml.) | | |
| 1 | Untreated control, compression | 5 | 45·2 (±2·7) | 47·8 (±3·1) | 49·2 (±1·9) | 49·2 (±2) | 6·65 (±0·22) | 7·17 (±0·14) | 7·55 (±0·21) | 7·41 (±0·13) | 5·3 (±0·5) | 7·9 (±0·33) | 7·9 (±0·33) | 0·14 (±0·04) | 0·13 (±0·02) | 0·17 (±0·03) | 2·3 (±0·4) | 1·9 (±0·07) | 2·1 (±0·03) |
| 2 | Chloralose, compression | 4 | 43·6 (±2·3) | 47·5 (±3·2) | 46·5 (±1·5) | 46·3 (±0·8 | 6·17 (±0·21) | 6·5 (±0·19) | 6·5 (±0·13) | 6·5 (±0·16) | 8 (±0·65) | 7 (±0·31) | 5·7 (±0·27) | 0·27 (±0·03) | 0·17 (±0·03) | 0·22 (±0·02) | 3·2 (±0·15) | 2·3 (±0·31) | 3 (±0·44) |
| 3 | PVP, no compression | 5 | 50·5 (±4·5) | 58·4 (±2·1) | 58·8 (±2·4) | 56 (±1·7) | 6·65 (±0·18) | 6·65 (±0·31) | 6·73 (±0·23) | 6·51 (±0·11) | 13·9 (±1·12) | 14·3 (±0·67) | 12·1 (±0·20) | 0·87 (±0·11) | 0·88 (±0·08) | 0·88 (±0·12) | 6·3 (±0·9) | 6·2 (±1·3) | 8·3 (±1·1) |
| 4 | PVP, compression .. | 5 | 50·2 (±2·8) | 58·5 (±3·5) | 59·7 (±3·1) | 59·6 (±1·6) | 6·91 (±0·46) | 7·12 (±0·26) | 6·94 (±0·10) | 6·61 (±0·10) | 14·4 (±0·95) | 15·4 (±0·32) | 15·8 (±0·82) | 0·93 (±0·1) | 1·09 (±0·62) | 1·25 (±0·18) | 6·6 (±0·23) | 7·7 (±0·81) | 9·2 (±0·27) |
| 5 | Chloralose, PVP, no compression | 4 | 44 (±3) | 59·7 (±3·9) | 61 (±2·7) | 60·2 (±2·7) | — | 6·1 (±0·14) | 6·3 (±0·37) | 6·3 (±0·12) | 25·6 (±1·7) | 27·4 (±3·22) | 27 (±2) | 1·53 (±0·29) | 1·56 (±0·17) | 1·53 (±0·09) | 6·1 (±0·15) | 5·8 (±1·2) | 5·8 (±0·38) |
| 6 | Chloralose, PVP, compression | 4 | 44 (±4) | 63·2 (±4·6) | 63·5 (±3·5) | 61·7 (±1·6) | 6·08 (±0·7) | 6·83 (±1·3) | 6·73 (±0·51) | 6·73 (±0·33) | 30·1 (±1·9) | 31 (±2) | 29·1 (±2·24) | 1·63 (±0·31) | 1·68 (±0·11) | 1·54 (±0·14) | 5·5 (±1·5) | 5·4 (±0·72) | 5·2 (±0·21) |
| 7 | Promethazine, PVP, compression | 5 | 44 (±2) | 45·5 (±1·5) | 46 (±3) | 43·3 (±2·7) | 6·67 (±1·9) | 6·63 (±1·22) | 7·11 (±0·40) | — | 2·5 (±0·15) | 3·7 (±0·4) | 2·6 (±0·1) | 0·18 (±0·06) | 0·26 (±0·03) | 0·21 (±0·06) | 5·92 (±0·3) | 5·8 (±0·17) | 4·7 (±0·62) |
| 8 | Promethazine, chloralose, PVP, compression | 5 | 44·4 (±1·6) | 46·4 (±2·4) | 48 (±2) | 46 (±3) | 7·3 (±0·5) | 7·2 (±0·19) | 7·25 (±0·32) | 7·1 (±1·1) | 4·6 (±0·2) | 4·6 (±0·31) | 2·9 (±0·09) | 0·29 (±0·02) | 0·30 (±0·02) | 0·20 (±0·11) | 5·10 (±0·25) | 6·6 (±0·18) | 5 (±0·5) |

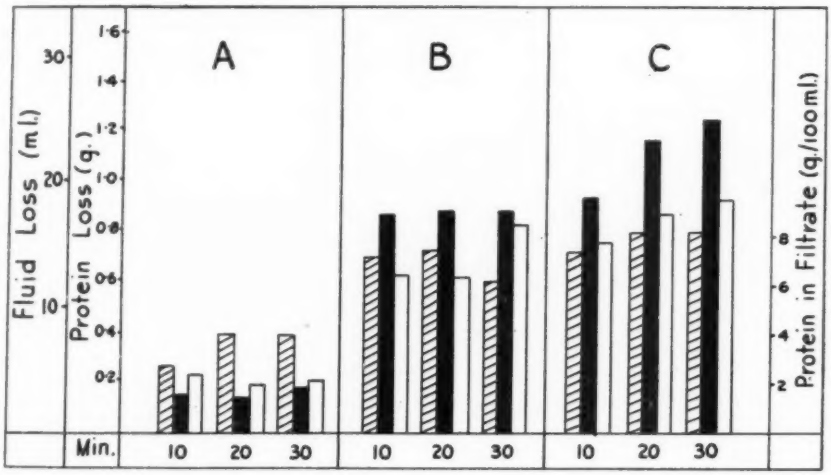


FIG. 1.—Effect of compression (A), of PVP (B), and of PVP combined with compression (C), on the permeability of the capillary wall to fluid, to protein, and on the capillary filtrate protein concentration, in unanaesthetized dogs. Hatched columns, fluid loss (ml.); filled columns, protein loss (g.); open columns, protein in filtrate (g./100 ml.).

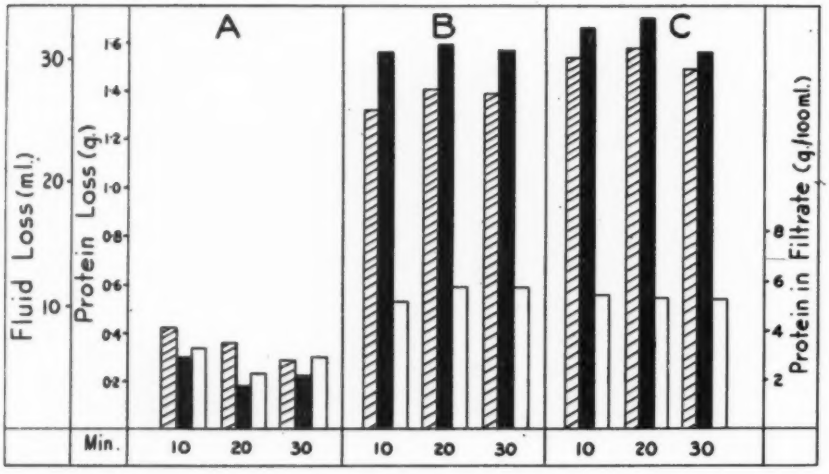


FIG. 2.—Effect of compression (A), of PVP (B), and of PVP combined with compression (C), on the permeability of the capillary wall to fluid, to protein, and on the capillary filtrate protein concentration, in dogs under chloralose. Hatched columns, fluid loss; filled columns, protein loss; open columns, protein in filtrate.

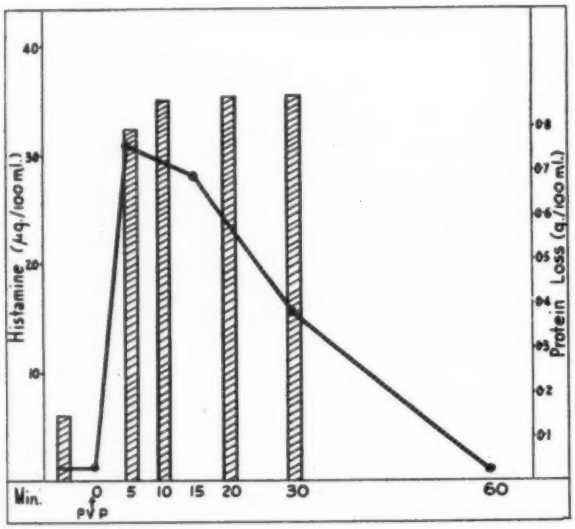


FIG. 3.—Plasma histamine level and capillary permeability to protein after injection of PVP to unanaesthetized dogs. Solid line, plasma histamine level in µg./100 ml.; hatched columns, protein loss in g./100 ml.

*Unanaesthetized Dogs, No Compression.*—The intravenous injection of PVP resulted in a significant increase in the extravascular filtration of water and of proteins through the capillary wall. The date summarizing these experiments are given in Table I, group 3, and the mean values are represented in Fig. 1 (division B).

The results indicate that, following an intravenous injection of PVP, a sudden increase in the permeability of the capillary wall, particularly to proteins, occurs. The filtration rate of proteins which, in normal dog, is an average of 0.17 g./100 ml. blood may increase to 0.88 g./100 ml. after PVP.

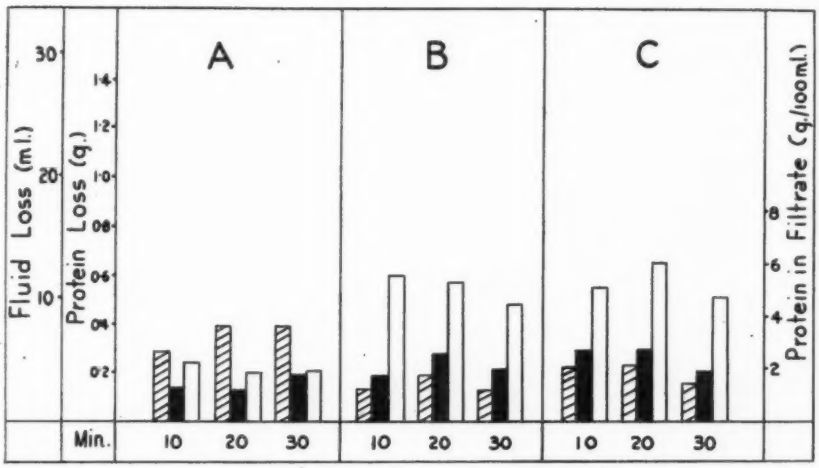


FIG. 4.—Action of promethazine on the permeability of the capillary wall to fluid, to protein, and on the capillary filtrate protein concentration, in unanaesthetized dogs treated with PVP (B), and with PVP combined with compression (C), in comparison with filtration rates in control animals (A). Hatched columns, fluid loss; filled columns, protein loss; open columns, protein in filtrate.

The significance of this difference is much greater, since in PVP-treated animals the figures were obtained without compression, whereas in the control animals a compression at 80 mm. Hg was applied for 30 min. A further consideration in this connexion is the finding that the increase in capillary permeability is correlated with the increase in plasma histamine concentration (Fig. 3). The sudden and massive release of histamine, which is partially reflected in the considerable increase in the plasma histamine concentration, is no doubt responsible for the alteration of the capillary function. The severe damaging action of PVP on the capillary wall becomes evident when one considers that the capillary filtrate contains a quantity of protein almost equal to that found in plasma.

*Unanaesthetized Dogs and Compression.*—Identical experiments have been performed with the addition of compression applied at the origin of the front leg.

As stressed above, the extent of the compression was variable, and was adjusted to the fluctuations of the arterial blood pressure produced by the injection of PVP, in order always to maintain a compression equal to half the value of the arterial blood pressure.

The results are reported in Table I, group 4, and the mean values are presented in Fig. 1 (division C).

A close analysis of the figures indicates that the addition of compression increases the rate of filtration by an average of 42% over the values found in the non-compressed animals.

*Anaesthetized Dogs, No Compression.*—These experiments were carried out to investigate whether anaesthesia with chloralose modifies the action of PVP on capillary permeability. We have noticed previously that PVP may cause death in animals under chloralose, whereas fatalities never occur in unanaesthetized dogs. Is the increase in the toxicity of PVP in anaesthetized animals related to some harmful effect of the anaesthetic on the capillaries? The experiments carried out on control dogs indicated that chloralose increases but slightly the protein filtration rate; nevertheless it might be different in animals undergoing shock by injection of PVP.

The mean figures obtained in this group of four animals are reported in Table I, group 5, and represented in Fig. 2, division B.

It is evident from these results that anaesthesia with chloralose increases significantly the extravasation of plasma proteins determined by the injection of PVP. The average increase, as compared with animals which received the same dose of PVP but no chloralose, is 73%.

The precise mechanism through which chloralose increases the toxic effects of PVP must still remain speculative.

*Anaesthetized Dogs and Compression.*—An attempt was made to amplify the noxious action of chloralose by the addition of compression to the administration of PVP.

In Table I, group 6, are summarized the mean data for these experiments, of which the values are represented in Fig. 2, division C.

These results indicate clearly that adding compression to anaesthesia with chloralose does not further increase the extravascular loss of plasma proteins caused by PVP. Apparently the capillary permeability has reached, in the previous conditions, the highest level which can no longer be increased by the addition of compression.

### *Action of Promethazine on the Capillary Permeability of PVP-treated Dogs*

If we accept that PVP determines the vascular disorders observed by a liberation of endogenous histamine, it involves, as a corollary, the inhibition

of these reactions by previous treatment with an antihistaminic drug. The choice of promethazine was justified by the previous findings that this drug is not only one of the most potent antihistamines, but also of very low toxicity (Halpern and Ducrot, 1946). In another series of studies, promethazine was shown to be the most powerful drug in preventing similar disturbances in rats treated with dextran (Briot and Halpern, 1952).

The dogs injected with promethazine were divided into two groups: the animals of the first group received PVP alone, whereas those of the second group received the same dose of PVP, while a compression was applied simultaneously at the origin of the leg. The level of compression was maintained invariably at 80 mm. Hg, as the arterial blood pressure does not drop noticeably following the injection of PVP in dogs treated with promethazine.

The experimental data reported in Table I, groups 7 and 8, and presented in Fig. 4 indicate clearly that the previous treatment with promethazine is highly efficient in preventing the disorders of capillary permeability induced by PVP. The action of the antihistaminic is evident in non-compressed as well as in compressed dogs. If the animals of the last group, which were submitted to the more severe test, are considered, it appears that the filtration rate of water is about the same and that of proteins hardly greater than the filtration rates observed in normal untreated animals to which the same compression was applied.

## Discussion

The data presented in this study provide evidence that the main feature of the action of PVP in dogs is a sudden and intense increase in the permeability of the capillaries to high-molecular substances, and particularly to plasma proteins, and that promethazine is able to inhibit the effects of PVP. Previous results indicate that PVP acts in dogs as a potent histamine liberator (Halpern and Briot, 1953b). The increase in the plasma histamine level, and the gastric hypersecretion (the latter not influenced by antihistamines) observed following the injection of PVP, present convincing evidence of this. It seems obvious that the released histamine from the cells accounts for the sudden alteration of the capillary permeability, which, in turn, elicits concomitant systemic disorders.

We should stress here that the technique described by Landis for determining the rate of capillary filtration can be used in dogs quite satisfactorily—provided that big dogs are selected—with some minor adjustments in the method to adapt it to the size of the animal. It is noteworthy that the protein filtration rate in dogs is very close to that found in man for about the same compression.

The comparison between the rate of simultaneous water and protein filtrations shows that both processes are not always parallel. These discrepancies affect, of course, the composition of the capillary filtrate. The independent filtration of these two constituents in pathological states has also been observed in man. It can be deduced from these observations that the speed and concentration at which water and crystalloids pass through the capillary wall may be different from the speed and concentration at which colloids and proteins pass through the same barrier.

As for the mechanism by which PVP deteriorates the capillary permeability, our opinion is that this action is due to the released histamine. Chambers and Zweifach (1942) and Zweifach (1953), using micromanipulative local injections, observed that histamine produces local arteriolar vasodilatation with consequent extravasation of plasma. There is evidence of changes in the appearance of the endothelial cells, and of oedema, with widespread stasis, developing rapidly. Stasis and increased permeability are aggravated by the constriction of the efferent venules produced by histamine in dogs. As compared with the observations of Zweifach, it seems that the disorders produced by the histamine liberator are more severe than those elicited by histamine itself. This is not surprising: histamine liberated at the cellular level must behave, in certain respects, differently from outwardly applied histamine: the local instantaneous concentration, the way of accosting the effectors, and the gradient of destruction, differ greatly. The same basic discrepancies have been observed with other humoral mediators, such as acetylcholine.

The loss of proteins which follows the injection of PVP is considerable, and reaches a rate, without any compression, about 5 to 6 times greater than that observed in normal dogs with compression. After the administration of PVP the capillary filtrate protein concentration reaches up to 80% of the plasma protein value. The application of compression in dogs treated with PVP does not increase significantly the protein concentration of the capillary filtrate.

The choice of the level of compression in animals treated with PVP raised some difficulties. A constant rate of compression at 80 mm. Hg would

involve an evident error, because PVP determines a drop of the arterial blood pressure, which usually stabilizes itself at a level lower than that of the compression. To keep the compression at a rate which is higher than the arterial blood pressure would tend to a complete arrest of local circulation, producing local ischaemia. It appeared to us that by adjusting the compression to the fluctuations of the arterial blood pressure, and maintaining it at half that of the blood pressure, the comparison remained valid.

Concerning the anaesthetic, it appears from the data reported that chloralose aggravates the damaging effect of the histamine liberator on the capillary wall. The results are rendered more valid by being obtained in the same animals at an interval of a few days. This aggravating action of chloralose on the permeability of the capillaries may explain the fatalities we observed in certain anaesthetized dogs after injection of PVP—accidents which never occur in unanaesthetized animals.

As to promethazine, its preventive action appeared constant and regular with or without anaesthesia. This action of the antihistaminic on the capillary permeability is parallel to its inhibitory action on the systemic symptoms of shock. Only the gastric hypersecretion caused by PVP is not influenced by the antihistaminic (Halpern and Briot, 1953b). But it should be remembered that gastric hypersecretion induced by histamine is not inhibited either by any known antihistaminic.

## Summary

1. The capillary filtration rates of water and protein determined in dogs with the Landis technique are very similar to those found in man.

2. The administration of polyvinylpyrrolidone (PVP) to dogs increases considerably the rate of protein and water filtration through the capillary wall. This effect is attributed to the release of endogenous histamine. Anaesthesia with chloralose increases the protein filtration rate in normal dogs only slightly, but potentiates the effect of PVP.

3. Promethazine inhibits the effect of PVP in unanaesthetized dogs as well as in those anaesthetized with chloralose.

The polyvinylpyrrolidone was kindly supplied by Specia-Rhône Poulenc, Paris. The work was supported in part by a grant from the Caisse Nationale de la Sécurité Sociale and Institut National d'Hygiène.

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# BRONCHOCONSTRICTOR AND BRONCHODILATOR ACTIONS OF ANTIHISTAMINE DRUGS

BY

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Among the many side actions of antihistamine drugs which have been reported, the only direct action on smooth muscle which is well known is that on the uterus ; most antihistamines have some oxytocic activity. It is generally believed that these drugs have no action of their own on the bronchial musculature, but a few exceptions have been reported. Bovet (1934) found that the early precursor thymoxyethyldiethylamine (929F) contracted guinea-pig bronchi, and Staub (1939) observed a similar action with *N*-phenyl-*N*-ethyl-*N'*-diethylethylenediamine (1571F). Castillo and de Beer (1947) show a tracing which illustrates that diphenhydramine ($10^{-5}$) contracts the guinea-pig tracheal chain, but do not comment thereon. Herxheimer (1953) found that inhalations of aerosols of 3% promethazine or 5% chlorcyclizine caused dyspnoea in guinea-pigs. On the other hand, De Schaepdrijver (1948) found that large doses of antazoline relaxed the bronchi of perfused guinea-pig lungs.

Antihistamines are usually tested against histamine, using only low concentrations of the antagonist. In order to antagonize the anaphylactic contraction of bronchial muscle, much higher concentrations are required. In the course of some work in this field it was observed that all the antihistamines used caused contractions of the guinea-pig tracheal chain in concentrations between $10^{-6}$ and $10^{-4}$, and above these concentrations they caused relaxation. Because these were the very concentrations required to antagonize the anaphylactic contraction of isolated guinea-pig trachea (Hawkins, 1952) or the allergic contraction of isolated human bronchus (Schild, Hawkins, Mongar and Herxheimer, 1951), the bronchoconstrictor action of antihistamine drugs was investigated further. The present work consists of a study of the direct action on the bronchial musculature of a series of twelve well-known antihistamine drugs.

## METHODS

*Guinea-pig Tracheal Chains.*—Tracheal chains (Epstein, 1932 ; Castillo and de Beer, 1947) were made using 6 to 8 rings, tied tightly with cotton thread, and orientated alternately so that the muscle strips were aligned. Guinea-pigs of all sizes were used, but it was found that chains were easiest to prepare from .the tracheas of animals weighing between 500 and 600 g. The preparations were maintained at 37° C. in Krebs-Henseleit solution (NaCl 6.87 ; KCl 0.43 ; $CaCl_2$ 0.28 ; $MgSO_4,7H_2O$ 0.29 ; $NaH_2PO_4,2H_2O$ 0.18 ; $NaHCO_3$ 2.10 ; glucose 1.00 g./l.) aerated with $O_2$ + 5% $CO_2$, or in a similar solution containing one-tenth as much $NaHCO_3$ aerated with $O_2$ alone. The tonus level of the preparations was continuously recorded with a light balsa-wood frontal writing lever giving a magnification of about ×20, tension about 200 mg., writing on a lightly smoked kymograph drum. The preparations were left for 1 to 2 hours before any drugs were given. During this period a marked rise of tonus level invariably occurred, the preparation then relaxing to a steady tonus level. Similar "post-mortem contractures" of isolated bronchial muscle were observed by Macht and Ting (1921).

In general, two or three submaximal doses of histamine, acetylcholine, or adrenaline were given, until regular responses were obtained, before any doses of antihistamine drugs were tested.

*Osmotic Change.*—In view of the rather high doses of drugs to be used, the effects of osmotic change were investigated. NaCl, added to the bath in concentrations up to $5 \times 10^{-4}$, had no distinct effect ; a concentration of $10^{-3}$, increasing the osmotic pressure by about 10%, produced a small fall in tonus, less than 5% of the maximum response to adrenaline. These results indicated that none of the responses to drugs described in the present work can be ascribed to effects on osmotic equilibria.

p*H Measurements.*—These were made electrometrically.

*Human Bronchial Chains.*—The methods described by Hawkins and Schild (1951) were used.

*Spinal Cats.*—The animals were anaesthetized with open ether, and the cord transected at the level of the

second cervical vertebra. The bronchial tone was recorded by the method of Konzett and Rössler (1940), and the common carotid pressure recorded in the usual way. Intravenous injections of drugs were made into the femoral vein and washed in with 2 ml. of saline.

*Bronchial Tone in Dogs.*—The animals were anaesthetized with ether followed by intravenous chloralose. The method of Konzett and Rössler (1940) was used for recording bronchial tone.

*Antihistamine Drugs.*—The drugs used were mepyramine acid maleate (Anthisan, May & Baker), promethazine hydrochloride (Phenergan, May & Baker), antergan hydrochloride (May & Baker), tripelennamine hydrochloride (Pyribenzamine, Ciba), antazoline hydrochloride (Antistin, Ciba), diphenhydramine hydrochloride (Benadryl, Parke, Davis), chlorcyclizine monohydrochloride (Di-Paralene, Abbott), phenindamine hydrogen tartrate (Thephorin, Roche), chloropyrilene citrate (Chlorothen, Lederle), methapheniline hydrochloride (Diatrin, William Warner), methapyrilene hydrochloride (Histadyl, Lilly), chlorprophenpyridamine maleate (Chlortrimeton, Schering).

I am grateful to the drug firms mentioned for supplies of the pure crystalline salts. In the present work drug concentrations are expressed in terms of the whole salt. The bath volume was 50 ml.

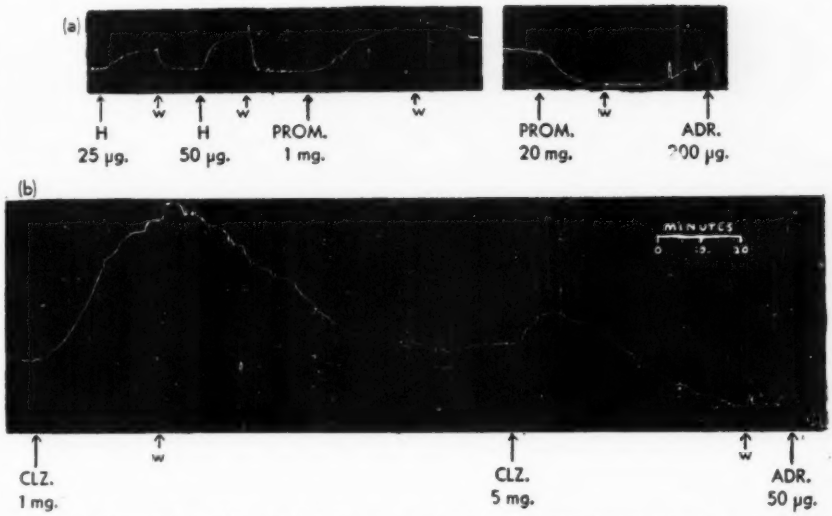


FIG. 2.—Guinea-pig tracheal chains in 50 ml. baths. (*a*) Bronchoconstrictor responses to histamine (H) and to promethazine (PROM): bronchodilator response to a large dose of promethazine. (*b*) Responses to chlorcyclizine (CLZ). ADR = maximum relaxation given by adrenaline.

## RESULTS

### *Guinea-pig Tracheal Chains*

Fig. 1 shows records from a guinea-pig tracheal chain. Following two responses to histamine, contractions produced by diphenhydramine are seen. The contractions in response to diphenhydramine $10^{-5}$ and $2 \times 10^{-5}$ are reversible, repeatable and graded. $2 \times 10^{-4}$ gave a very little larger contraction than did $2 \times 10^{-5}$; $8 \times 10^{-4}$ gave a small contraction and then relaxation.

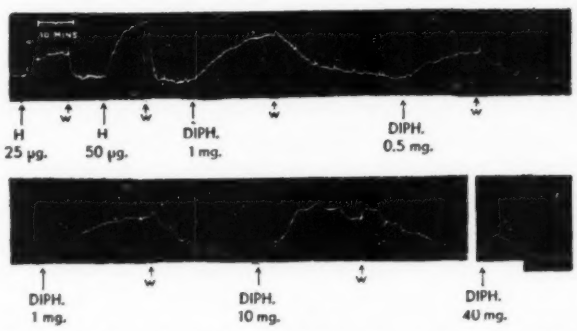


FIG. 1.—Guinea-pig tracheal chain in 50 ml. bath. Bronchoconstrictor responses to histamine (H) and to diphenhydramine (DIPH).

Fig. 2 shows similar effects with promethazine and chlorcyclizine. With promethazine, $2 \times 10^{-5}$ contracted the trachea, whereas $4 \times 10^{-4}$ produced a relaxation which was as great as a maximal relaxation caused by adrenaline. On another preparation, chlorcyclizine ($2 \times 10^{-5}$) gave a big contraction, while chlorcyclizine ($10^{-4}$) produced a small contraction followed by a nearly maximal relaxation. Similar effects were given by mepyramine, methapyrilene, tripelennamine, phenindamine, chlorprophenpyridamine, antergan, chloropyrilene, methaphenilene, and antazoline.

The actions of these twelve antihistamine compounds, in concentrations from $10^{-7}$ to $10^{-3}$, were studied on some 140 preparations from 64 guinea-pigs. Fig. 3 shows the concentrations at which this series of antihistamines have actions of their own on guinea-pig trachea. Within the range of concentrations $10^{-6}$ to $10^{-4}$ they all gave contractions. The compounds in Fig. 3 are arranged in order according to the magnitude of the range over which bronchoconstrictor responses were recorded. The bronchoconstrictor action seems to be a common property of antihistamine drugs and there is little to choose in this respect between mepyramine, diphenhydramine, methapyrilene, promethazine, and tripelennamine, the first five compounds in Fig. 3. Higher doses of all the compounds, within the range of $10^{-4}$ to $10^{-3}$, gave relaxations,

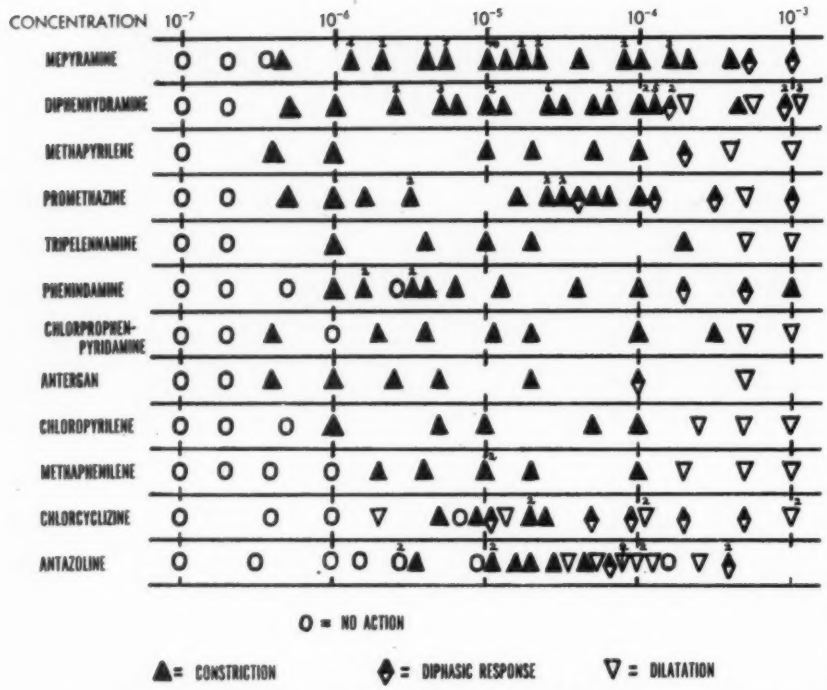


FIG. 3.—Concentrations (logarithmic scale) of antihistamine drugs giving bronchoconstrictor and bronchodilator responses in a series of guinea-pig tracheal preparations. Where more than one response is represented by a single point, the number of responses is indicated.

and intermediate doses often gave diphasic responses, an initial contraction being followed by relaxation to below the resting baseline (Fig. 3).

The only clear difference between the compounds shown in Fig. 3 seems to be with antazoline and chlorcyclizine, where the bronchoconstrictor action was less pronounced, and the relaxing action occurred at lower concentrations. The minimum concentrations of the drugs active in causing bronchoconstriction in guinea-pig tracheal chains were estimated and are shown in Table I. The method of Kärber (Gaddum, 1933) was used to estimate these concentrations. In addition, the lowest concentration which produced bronchodilatation was estimated for each drug, and also the range of concentrations over which the drug exhibited bronchoconstrictor activity. These values are also shown in Table I.

It will be seen from Table I that all the compounds are active bronchoconstrictors, but that chlorcyclizine and antazoline are the least active. Mepyramine, diphenhydramine, and methapyrilene have bronchoconstrictor activity over a thousandfold or greater range of concentrations; the range with chlorcyclizine and antazoline is very limited. Antazoline is the only compound in the series which can cause bronchodilatation in a concentration of less than $10^{-4}$.

TABLE I

CONCENTRATIONS OF ANTIHISTAMINE DRUGS HAVING DIRECT ACTIONS ON GUINEA-PIG TRACHEA

| Drug | Minimum Active Concn. for Broncho-constriction (μg./ml.) | Minimum Active Concn. for Broncho-dilatation (μg./ml.) | Range of Concentrations Having Broncho-constrictor Activity |
|---|---|---|---|
| Mepyramine .. | 0·4 | 670 | 1,700 |
| Diphenhydramine | 0·3 | 410 | 1,300 |
| Methapyrilene .. | 0·2 | 200 | 1,000 |
| Promethazine .. | 0·3 | 230 | 730 |
| Tripelennamine .. | 0·4 | 320 | 710 |
| Phenindamine .. | 0·9 | 440 | 510 |
| Chlorprophen-pyridamine .. | 1·1 | 400 | 370 |
| Antergan .. | 0·3 | 100 | 350 |
| Chloropyrilene .. | 0·8 | 160 | 200 |
| Methaphenilene | 1·4 | 140 | 100 |
| Chlorcyclizine .. | 4·7 | 110 | 22 |
| Antazoline .. | 5·3 | 41 | 8 |

The direct actions of antihistamine drugs on bronchial muscle appear to be independent of their antihistamine activities. For example, among the drugs in the series most active in antagonizing histamine are mepyramine, promethazine, and chlorcyclizine; mepyramine and promethazine are active bronchoconstrictors whereas chlorcyclizine is not nearly so powerful in this respect. Of the weaker drugs in antagonizing histamine, diphenhydramine and antergan are active bronchoconstrictors over a wide range of concentrations, whereas antazoline is the weakest of the series as a bronchoconstrictor. The specificity of the antihistamine drug does not appear to influence the bronchoconstrictor activity—for example, both mepyramine and diphenhydramine are active bronchoconstrictors, mepyramine being a highly specific antihistamine, while diphenhydramine has a marked anti-acetylcholine action (Schild, 1947).

In the same way there is no obvious relation between antihistamine activity and bronchodilator activity on the tracheal chain.

*Action of Atropine.*—The bronchoconstrictor response to mepyramine, $10^{-5}$, was not inhibited by atropine, $10^{-5}$ (Fig. 4a).

. As atropine itself has a weak antihistamine action on the tracheal chain, this drug was also tested in high concentrations. Though it had no action in a concentration of $10^{-5}$, at $10^{-4}$ it gave a marked contraction (Fig. 4b).

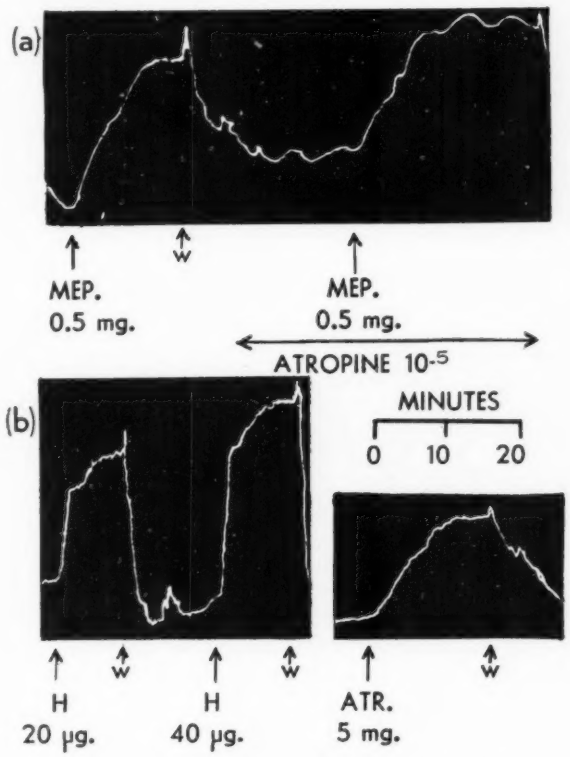


FIG. 4.—Guinea-pig tracheal chains. Bath vol. 50 ml. (*a*) Response to mepyramine ($10^{-5}$) not reduced by atropine ($10^{-5}$). (*b*) Bronchoconstrictor responses to histamine (H) and atropine (ATR).

*Secondary Relaxing Action of Histamine on Guinea-pig Trachea.*—In view of the observation of Arunlakshana (1953) that diphenhydramine and antazoline can release relatively large amounts of histamine from guinea-pig and human lung tissue, it was considered of interest to examine the actions of high concentrations of histamine on the tracheal chain, in the absence and presence of antihistamine drugs.

Fig. 5a shows that, following a maximal contraction produced by the action of histamine, $2 \times 10^{-5}$, addition of further large doses of histamine to the bath produced relaxation. This secondary relaxing action of high doses of histamine is reminiscent of the secondary depressant action of the drug on the intestine (Olivecrona, 1921).

It was also found that in the presence of sufficient diphenhydramine to abolish completely the contracting action of histamine a high dose of

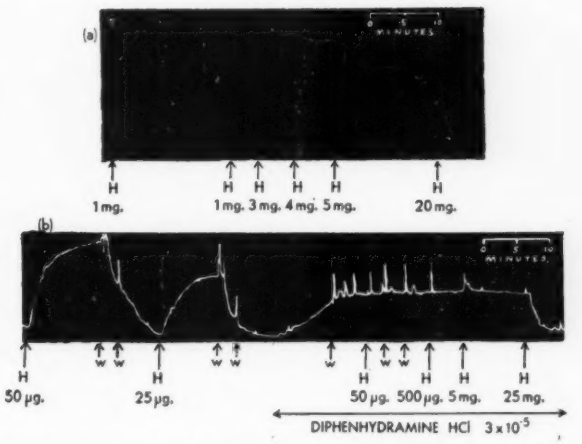


FIG. 5.—Guinea-pig tracheal chains. Bath vol. 50 ml. (*a*) Maximal contraction produced by histamine (H); further large doses of histamine gave relaxation. (*b*) Diphenhydramine ($3 \times 10^{-5}$) abolished the bronchoconstrictor response to all doses of histamine; in the presence of diphenhydramine, a large dose of histamine ($5 \times 10^{-4}$) produced relaxation.

histamine ($5 \times 10^{-4}$) produced relaxation of the trachea (Fig. 5b). Similar results were obtained in the presence of antazoline ; the phenomenon has also been observed with human bronchi (Hawkins, 1952). The secondary relaxing action of histamine on bronchial muscle may be related to the relaxing action on the rat's uterus ; Gordonoff (1944) has shown that the latter effect of histamine is not antagonized by antergan ; Dews and Graham (1946) obtained a similar result with mepyramine.

*Guinea-pig Ileum.*—Feldberg and Smith (1954) have shown that very small doses of mepyramine can stimulate guinea-pig ileum preparations, particularly after the preparation has been in contact with the histamine releaser 48/80, and have attributed this effect to histamine release by mepyramine. Some support is given to this view by the observation of Mongar and Schild (1952) that previous contact with 48/80 also very much increases the amount of histamine released by guinea-pig ileum in anaphylaxis.

The present observations were confined to high doses of antihistamine drugs. It was found that mepyramine, diphenhydramine, promethazine, phenindamine and antergan, in concentrations of $10^{-4}$, all caused slow contractions of guinea-pig ileum preparations. No such action was observed with chlorcyclizine or antazoline in the same concentrations.

*pH Measurements.*—All the antihistamine salts used produced bronchoconstriction in concentrations which gave no detectable change in the *p*H of the bath fluid. It was considered possible that the highest concentrations tested, which were found

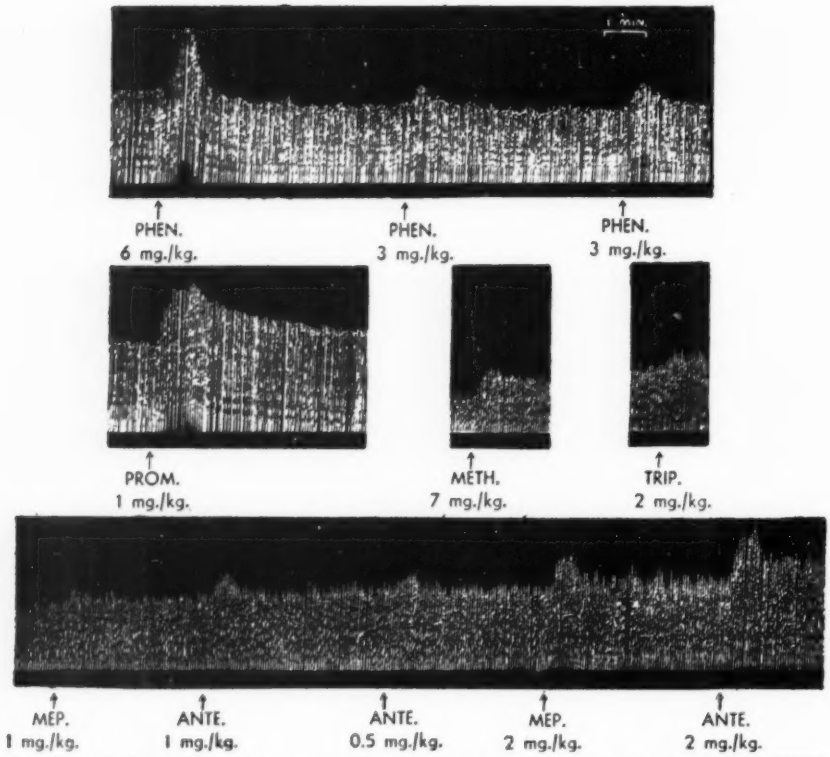


FIG. 6.—Spinal cats, bronchial tone. Bronchoconstrictor responses to phenindamine (PHEN), promethazine (PROM), methapyrilene (METH), tripelennamine (TRIP), mepyramine (MEP), and antergan (ANTE), injected i.v.

to give relaxation of the tracheal chains, might produce effects by altering the *p*H. It was found that the *p*H of the bath fluid could be changed within the range 10.9 to 5.1, by addition of NaOH or HCl, without producing any response of the tracheal chains. With most of the antihistamine salts the highest concentrations used produced only trivial changes of *p*H, within this range, but with phenindamine acid tartrate ($10^{-3}$) and chloropyrilene citrate ($10^{-3}$) the *p*H was 4.6. Since these doses produced relaxation, while alterations of the bath *p*H to 1.7 with HCl gave only a small contraction, it is evident that the bronchodilator responses observed with high doses were not a consequence of *p*H change.

The highest concentration of histamine acid phosphate tested was $10^{-3}$, which gave a bath *p*H of 5.1. Addition of HCl to the bath to give this *p*H had no action on the preparation, under the experimental conditions.

*Spinal Cats and Anaesthetized Dogs*

The finding that antihistamines produce bronchoconstriction was confirmed in spinal cats and dogs under chloralose. The bronchial tone was recorded by Konzett's method and drugs were injected intravenously. Fig. 6 shows bronchoconstrictions, indicated by a rise in the record, produced by phenindamine, promethazine, metha-

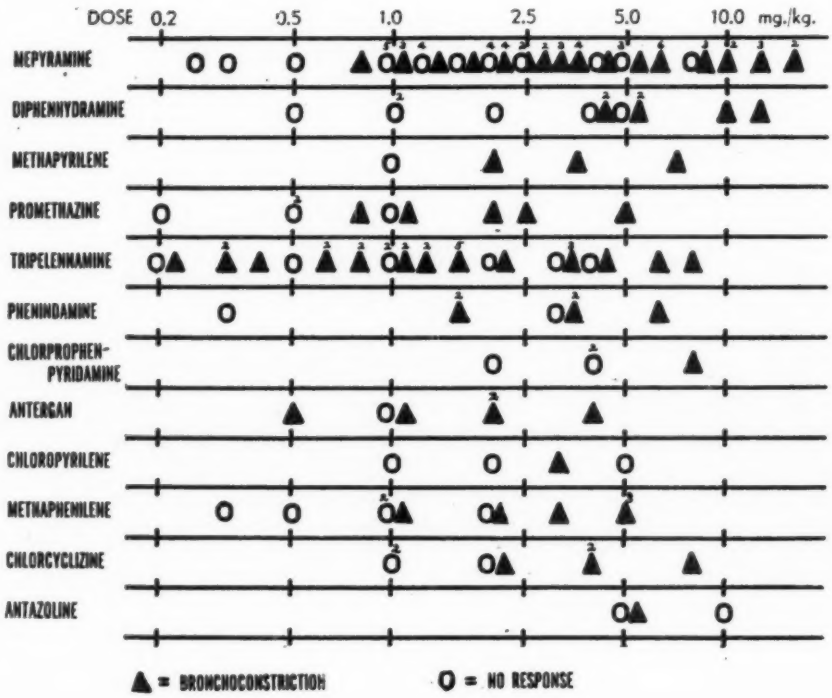


FIG. 7.—Spinal cats; bronchoconstrictor responses to antihistamine drugs injected i.v. Logarithmic scale of doses. Where more than one response is represented by a single point, the number of responses is indicated.

pyrilene, and tripelennamine in spinal cats. A comparison of antergan with mepyramine is also shown, and it will be seen that antergan is a more active bronchoconstrictor than mepyramine in the spinal cat.

The responses from 18 cats are summarized in Fig. 7. All the compounds gave constrictions in doses within the range 1.0 to 10.0 mg./kg. The compounds are arranged in the same order as in Fig. 3. Chlorcyclizine and antazoline have only a feeble bronchoconstrictor action in the spinal cat.

Some responses of the bronchi in dogs under chloralose are shown in Fig. 8. In this experiment, bronchoconstrictor responses to promethazine were compared with similar responses to mepyramine. As in the spinal cats, the bronchoconstrictions were accompanied by depressor responses, and simultaneous recordings of arterial pressure were made. The experiment illustrated in Fig. 8 showed that, although mepyramine and promethazine were approximately equiactive as bronchoconstrictors in the anaesthetized dog, the latter drug was twice as active as the former in lowering the blood pressure. The results of a number of similar experiments, comparing the simultaneously recorded bronchoconstrictor and depressor responses to mepyramine with those to other antihistamine drugs, are given in Table II. It will be seen that there is no correlation between the relative bronchoconstrictor activities and the relative depressor activities determined at the same time. For example, chloropyrilene was as active on the blood pressure as mepyramine, but the former had less than one-fifth of the activity of the latter on the bronchi. Dose levels which were found to be active on the bronchi are included in Table II.

The antihistamine compounds thus have the common property of causing bronchoconstriction in the spinal cat and anaesthetized dog. It seems likely that this is a direct action on the bronchial muscle, comparable with that found with guinea-pig trachea. It was seen with all the compounds at dose levels which are comparable in the isolated bronchial muscle and in the whole animal, $10^{-6}$ to $10^{-4}$ in the guinea-pig tracheal chain, and 1 to 10 mg./kg. in the spinal cat or anaesthetized dog. Further, though in general direct comparisons of activity were not made in the guinea-pig trachea or spinal cat, the relative bronchoconstrictor activities of antergan and mepyramine were in fact determined on all three preparations. The activity ratios were, as antergan/mepyramine: guinea-pig trachea, 1.4; spinal cat, 1.5; anaesthetized dog, 1.4.

TABLE II

BRONCHOCONSTRICTOR AND DEPRESSOR ACTIVITIES OF ANTIHISTAMINE DRUGS IN ANAESTHETIZED DOGS

| Drug | Doses having a Broncho-constrictor Action (mg./kg.) | Relative Potencies (Mepyramine = 100) | |
|---|---|---|---|
| | | Broncho-constrictor Activity | Depressor Activity |
| Mepyramine .. | 1·0 | 100 | 100 |
| Diphenhydramine | 2·0 | 45 | 20 |
| Methapyrilene .. | 2·0 | 45 | 125 |
| Promethazine .. | 1·0 | 90 | 180 |
| Tripelennamine .. | 1·0 | 100 | 165 |
| Phenindamine .. | 1·0 | 100 | 125 |
| Antergan .. | 1·0 | 140 | 200 |
| Chloropyrilene .. | 5·0 | <20 | 105 |
| Methaphenilene .. | 2·0 | — | 150 |

*Bronchodilator Action in Spinal Cats and Anaesthetized Dogs.*—In the spinal cat, bronchoconstrictor responses were obtained rather irregularly (Fig. 7). It was found that animals which failed to give bronchoconstrictor responses initially often gave them regularly after repeated doses. In addition it was noted that animals which had suffered considerable trauma or delay in the operative procedure gave bronchoconstrictor responses more readily than did animals which showed no evidence of shock after transection of the spinal cord. At the same time it was often observed that the first dose or two of antihistamine gave a long-lasting pressor response

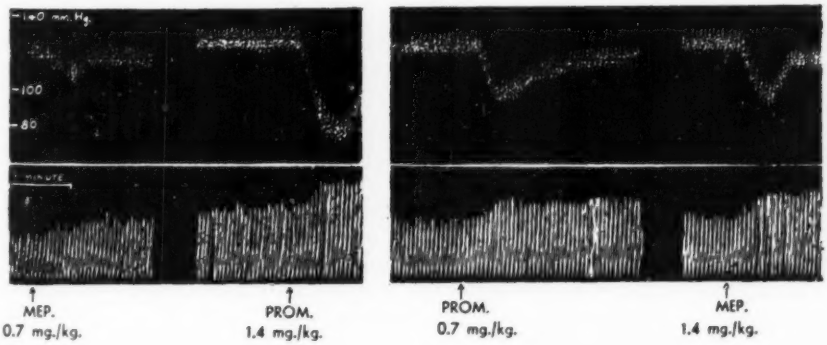


FIG. 8.—Anaesthetized dog. Bronchoconstrictor and depressor responses to mepyramine (MEP) and promethazine (PROM) injected i.v.; 10 min. intervals between doses.

initially which showed tachyphylaxis, becoming depressor after repeated doses.

Fig. 9 shows this effect with mepyramine ; similar results were obtained with the other antihistamines used. The upper tracing in Fig. 9, a record

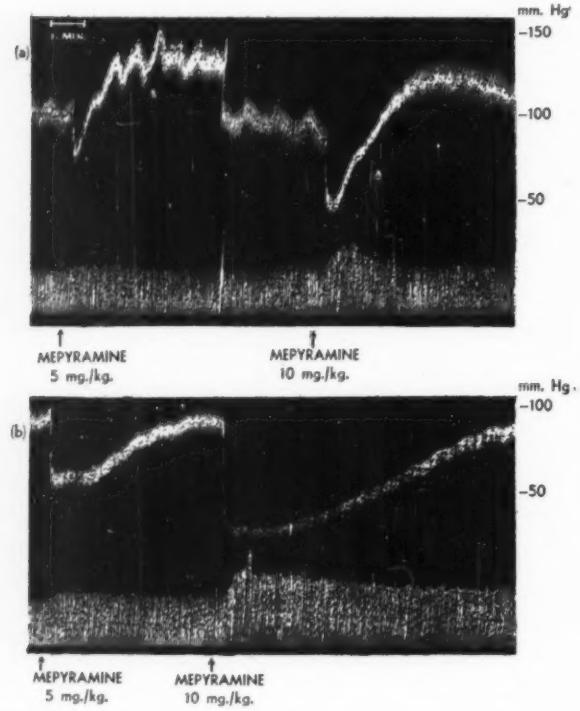


FIG. 9.—Spinal cat; arterial pressure and bronchial tone. Responses to i.v. mepyramine. 45 min. interval between (*a*) and (*b*), with doses of mepyramine at 10 min. intervals.

of the bronchial tone and blood pressure of a spinal cat, shows that initially the response to mepyramine was largely pressor. Three-quarters of an hour later, after repeated doses, the blood-pressure response had become purely depressor, while the bronchial responses were more prolonged.

In one or two animals more extreme changes were obtained, the records showing either diphasic responses consisting of an initial bronchoconstriction followed by prolonged dilatation, or at times a simple long-lasting bronchodilatation, in the early stages of the experiment. These bronchodilatations were tachyphylactic, and after repeated doses pure bronchoconstrictions were obtained. These changes were always paralleled by the blood-pressure responses, bronchodilatations being accompanied by pressor, and bronchoconstrictions by depressor, responses.

*Human Bronchi*

*Isolated Human Bronchial Chains.*—Actions of antihistamine drugs, similar to those observed on the guinea-pig tracheal chain, were found with preparations of human bronchi. It has previously been shown (Hawkins and Schild, 1951 ; Schild *et al.*, 1951) that mepyramine will contract human bronchi. In the course of the present work it was found that this drug in concentrations from $4 \times 10^{-6}$ to $8 \times 10^{-5}$ caused contraction of human bronchial chains, whereas a concentration of $4 \times 10^{-4}$ caused relaxation.

High concentrations of promethazine, of the order of $4 \times 10^{-5}$, caused relaxation. Antazoline was inactive in a concentration of $10^{-6}$, but gave a small relaxation at $8 \times 10^{-4}$.

## DISCUSSION

It has been shown that 12 well-known antihistamines possess bronchoconstrictor actions when tested on the guinea-pig tracheal chain in concentrations greater than $10^{-6}$. The drugs in question have a bronchoconstrictor action when injected intravenously into spinal cats or anaesthetized dogs at dose levels which are comparable to those which are required to contract the guinea-pig trachea. Further, similar results were obtained with isolated human bronchial muscle preparations.

This bronchoconstrictor action seems to be a common property of antihistamine drugs. It is least marked with antazoline and chlorcyclizine, and it is interesting to note the structural feature, not possessed by the other compounds in the series, which these two drugs have in common. The basic structure of an antihistamine of the ethylenediamine series consists of aromatic or heterocyclic rings attached to one of the nitrogen atoms, while the other nitrogen atom is generally substituted by aliphatic groups. The principal modification of this structure possessed in common by antazoline and chlorcyclizine is the inclusion of the "aliphatic *N*" in a heterocyclic ring, the imidazoline ring in antazoline, and the piperazine ring in chlorcyclizine. This cyclization of the aliphatic end of the antihistamine molecule might be responsible for modifying the bronchoconstrictor action and enhancing the direct bronchodilator action seen with higher doses.

*Mode of Action.*—The bronchoconstrictor action of mepyramine was not inhibited by atropine, and it seems unlikely that this action is parasympathomimetic.

An alternative possibility is that antihistamine drugs possess a "histamine-like" action. Such

actions of antihistamines on smooth muscle as have been described show some parallelism with the actions of histamine. Oxytocic actions on the uterus of the guinea-pig, rabbit, or dog have been demonstrated for antergan, mepyramine, methapyrilene and tripelennamine (Halpern, 1942; Bovet and Walthert, 1944; Lee, Dinwiddie and Chen, 1947; Sherrod, Loew and Schloemer, 1947) and histamine contracts these organs. Scudi, Reinhard, and Dreyer (1948) found that thonzylamine stimulated the isolated rat uterus. Though it is commonly believed that histamine always relaxes this organ, Feldberg (1941) has pointed out that under certain conditions histamine will stimulate it.

On the intestine, Sherrod *et al.* (1947) showed that mepyramine and tripelennamine stimulated the dog's small intestine. Scudi *et al.* (1948) and Dreyer (1950) found that though small doses of thonzylamine caused a transient inhibition of the contractions of the cat's small intestine, *in situ*, high doses frequently caused stimulation. The responses persisted after cutting the splanchnic and vagus nerves. Dreyer (1950) mentions similar results with mepyramine, but found that prophenpyridamine gave stimulation in small doses, larger doses causing depression. Feldberg and Smith (1954) have recently shown that very small doses of mepyramine can not only potentiate the action of histamine on isolated guinea-pig ileum but will, under certain conditions, stimulate this preparation directly. It was found in the present experiments that high concentrations of some antihistamines contracted guinea-pig ileum.

Further actions in which antihistamines resemble histamine are in producing a fall in blood pressure, in causing vasodilatation in the perfused dog's leg (Murray and Huston, 1952), and on the salivary glands in dogs, where antergan causes secretion (Halpern, 1942); in the course of the present experiments on dogs it was noted that the large doses of antihistamines injected caused salivation. In addition it has been reported that diphenhydramine will directly stimulate gastric secretion in man (Doran, 1947; Gilg, 1948), and a number of antihistamines have been found to potentiate histamine-induced gastric secretion (Deutsch, 1947; Emmelin and Frost, 1947; Wood, 1948; Ashford, Heller, and Smart, 1949; Dutta, 1949; Howat and Schofield, 1954).

Whereas histamine in moderate doses causes the guinea-pig tracheal chain to contract, it has been shown that very large doses have a depressant action (Fig. 5). The actions of antihistamines which have been demonstrated on the guinea-pig trachea are therefore similar to those of histamine. In the same way, it may be demonstrated, using the Konzett preparation, that histamine has a bronchoconstrictor action in cats and dogs.

There is thus a considerable degree of parallelism between the independent actions of antihistamines and the actions of histamine itself. It seems likely that the constrictor and dilator actions of moderate and high concentrations, respectively, of antihistamines on bronchial muscle are in fact histamine-like actions.

The possible mechanism of these histamine-like actions of antihistamines may be one of the following: (*a*) A histamine-releasing action, the antihistamine releasing or activating cellular histamine in the tissue concerned. (*b*) An antihistaminase action, resulting in potentiation of the action of small amounts of free histamine in the tissues to such a degree that recognizable effects are produced. (*c*) A truly "mimetic" action, from the structural similarity between the antihistamine drugs and histamine itself being sufficiently marked for high concentrations of antihistamines to stimulate "histamine receptors" in the tissues.

It has in fact been reported that antergan, mepyramine, and the antihistamine compound 2325 R.P. cause marked elevation of the blood histamine when administered orally or intravenously in man (Pellerat and Murat, 1946). Further, Arunlakshana (1953) has shown that diphenhydramine and antazoline release quite large amounts of histamine from guinea-pig or human lung tissue. If it is assumed that the pharmacological action of the intrinsic histamine released is relatively resistant to antagonism by antihistamines, the mode of action of antihistamine drugs on smooth muscle may be interpreted as follows. With the lowest concentrations of antihistamines (less than $10^{-6}$), the amounts of histamine released are so small, except in certain special circumstances such as after administration of compound 48/80 (Feldberg and Smith, 1954), as to be inactive. With concentrations of the order $10^{-6}$ to $10^{-4}$, active amounts of intrinsic histamine, relatively resistant to the antihistamine action, are released, resulting in contraction of the muscle. With the highest concentrations of antihistamine (greater than $10^{-4}$) sufficient antihistamine activity may be present to prevent any stimulant action of intrinsic histamine, revealing the depressant action of the relatively high concentrations of histamine released locally.

Atropine was also found to have a bronchoconstrictor action in high concentrations; this drug has been shown to release histamine (Burstein and Parrot, 1949; Schacter, 1952).

It is unlikely that the weak antihistaminase action of promethazine and antazoline (Kapeller-Adler, 1949) is related to their bronchoconstrictor actions, since mepyramine, diphenhydramine, and antergan, which possess bronchoconstrictor activity, do not block histaminase. Further, highly active antihistaminase drugs do not contract guinea-pig tracheal chains (Arunlakshana, Mongar, and Schild, 1954).

The available evidence would therefore seem to be compatible with the view that the independent actions of antihistamines are, at least in part, attributable to their histamine-releasing activity. The possibility that the antihistamines may in fact stimulate the same receptors as histamine must also be borne in mind.

*Antihistamines and Anaphylaxis in Isolated Bronchial Muscle.*—The range of concentrations over which the majority of antihistamines possess bronchoconstrictor activity ($10^{-6}$–$10^{-4}$) is in fact the range over which these drugs show some activity in antagonizing the anaphylactic bronchoconstriction in isolated tracheal chains from sensitized guinea-pigs (Hawkins, 1952). Further, as Schild *et al.* (1951) have shown, concentrations of mepyramine sufficient to reduce the allergic bronchoconstriction in isolated bronchi from asthmatic patients, are also sufficient to cause bronchoconstriction.

Concentrations of antihistamines sufficient to produce bronchodilatation (Table I, Fig. 3) could probably not be achieved in the intact animal, owing to the toxicity of the drugs. Hence the bronchoconstrictor activity of the antihistamine drugs tested limits their usefulness in counteracting anaphylactic or allergic bronchospasm.

*Relation to the Therapy of Asthma.*—Most authorities now hold the view that antihistamine drugs have little value in the relief of bronchospasm in severe asthma, though some authors have found the usual therapeutic doses of the drugs useful in controlling relatively mild attacks of asthma. When efforts have been made to secure higher local concentrations of these drugs, with a view to suppressing severe asthma, bronchoconstriction has been observed. Charlier and Philippot (1949) have shown that antergan or mepyramine, administered as 2% aerosols, can cause bronchoconstriction in man; antazoline, as a 5% aerosol, was inactive. Herxheimer (1952) records that inhalation of aerosols containing antihistamines in concentrations greater than 1 to 3% invariably aggravated the dyspnoea of asthmatic patients.

In addition, a number of authors have reported that ingestion of the usual therapeutic doses of antihistamines by asthmatic and other patients resulted in symptoms suggestive of bronchoconstriction. Levy and Seabury (1947) and Criep and Aaron (1948a and b) found that oral administration of antihistamine drugs caused some reduction of the vital capacity in a proportion of asthmatic patients.

It therefore seems reasonable to conclude that the bronchoconstrictor action of antihistamine drugs may be in part responsible for the failure of the drugs to relieve severe asthma. It would appear to be desirable to develop potent antihistamine compounds which do not possess bronchoconstrictor activity.

## Summary

1. A series of 12 well-known antihistamine drugs has been found to possess bronchoconstrictor activity in concentrations within the range $10^{-6}$ to $10^{-4}$ when tested on isolated guinea-pig tracheal chains, and in doses within the range 1 to 10 mg./kg. when injected intravenously into spinal cats or anaesthetized dogs.

2. There was no correlation between the relative antihistamine and bronchoconstrictor activities of the antihistamine drugs tested.

3. Three compounds, mepyramine, diphenhydramine, and methapyrilene, caused bronchoconstriction in over a thousandfold range of concentrations; chlorcyclizine and antazoline had only small ranges of bronchoconstrictor activity. Promethazine, tripelennamine, phenindamine, chlorprophenpyridamine, antergan, chloropyrilene, and methaphenilene were intermediate with respect to range of bronchoconstrictor activity.

4. In higher concentrations, $>10^{-4}$, the antihistamine drugs tested caused relaxation of isolated guinea-pig tracheal chains.

5. Similar results with mepyramine, promethazine, and antazoline were obtained using isolated human bronchial chains.

6. Atropine $10^{-5}$ did not antagonize the bronchoconstrictor effect of mepyramine $10^{-5}$; atropine $10^{-4}$ itself caused contraction of guinea-pig tracheal chains.

7. It is suggested that the direct actions of antihistamine drugs on bronchial muscle are "histamine-like" actions, and may be due, at least in part, to the histamine-releasing properties of the drugs.

8. It is concluded that the bronchoconstrictor action of the antihistamine drugs tested may well be a factor limiting their therapeutic activity in asthma.

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# A STUDY OF TWO METHODS FOR TESTING LOCAL ANAESTHETICS IN MAN

BY

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Several methods are available for assessing local anaesthetic activity. For terminal anaesthesia the most widely used quantitative method is probably that based on intradermal wheals on the shaved backs of guinea-pigs (Bülbring and Wajda, 1945). The first part of this paper reports results obtained by a similar method on man. These results were at least as accurate as those obtainable on the guinea-pig, but the activity ratios found in the two species were different.

In the second part of the paper a further method for comparing the activity of local anaesthetics is described, based on an observation by Bert (1885) that, although cocaine had no effect on normal skin, it readily anaesthetized the base of an excised blister. A blister is formed on the flexor surface of the forearm using a cantharidin plaster (Armstrong, Dry, Keele, and Markham, 1953). A number of solutions are tested in succession on this exposed area of dermis. By this method an assay can be carried out which is as accurate when used on a single subject as is the wheal method on 20 subjects. When the assay is repeated the results agree within close limits. The activities of cinchocaine, lignocaine (lidocaine), cocaine, butacaine, amethocaine and "ravocaine" (2-propoxyprocaine) relative to procaine have been tested by this method.

## METHODS

*Intradermal Wheal in Man.*—Most of these experiments were carried out during the course of routine practical classes for Second M.B. students. Each student was injected on the flexor surface of the forearm with four or six wheals (0.2 ml.) containing different concentrations of two local anaesthetic drugs in saline. The students were tested in groups of six to nine and the results from a number of groups pooled. The doses were arranged in random order along the forearm to allow for differences in sensitivity. To avoid subjective errors of assessment, the wheals were identified with letters until testing had been completed. Each wheal was tested for anaesthesia by pricking six times in different places with a pin, using a stimulus just great enough to elicit pain on normal skin near the wheal. The number of pricks not felt gave a measure of the degree of anaesthesia. The test was repeated every five minutes for half an hour and the summated response used as a measure of activity. The combined results of tests done at a particular time after the injection were used to study the time course of anaesthesia.

*Test on Exposed Dermis in Man.*—In this method an exposed area of dermis on the flexor surface of the forearm was used for comparing the activity of local anaesthetics in man. The epidermis was conveniently removed by employing the vesicant action of cantharidin. A 0.3% cantharidin plaster was applied to the area for 6 to 8 hr. (Armstrong *et al.*, 1953). A blister is formed which appears to leave the network of nerve endings in the dermis relatively intact. Only a small area need be exposed for this test—about ¼ sq. cm. After excision of the blister the area was bathed alternately in a stream of Tyrode solution and local anaesthetic solution, using the arrangement shown in Fig. 1. The solutions flowed at a constant rate of about 10 ml./min. from reservoirs through a jacketed warming-tube on to the exposed area. A mechanical stimulus was applied with the spring-loaded camel-hair brush shown in the inset of Fig. 1. The bristles form a plunger which moves into the outer casing as the load on the brush is increased. The outer casing carries a scale, and the load on the brush is indicated by the position of the end of the plunger relative to this scale. The bristles were placed lightly on the exposed area and the load increased until a definite pricking sensation was felt as the individual hairs came into contact with the unanaesthetized dermis. The scale reading at this load was noted and was kept constant throughout the assay. The position of the brush was then changed slightly and the stimulus repeated twice at intervals of about 2 sec. This stimulus is the base-line from which anaesthesia is measured. It was repeated each time before an anaesthetic solution was applied. Certain precautions must be taken to ensure constancy of the stimulus throughout the assay : the brush must not

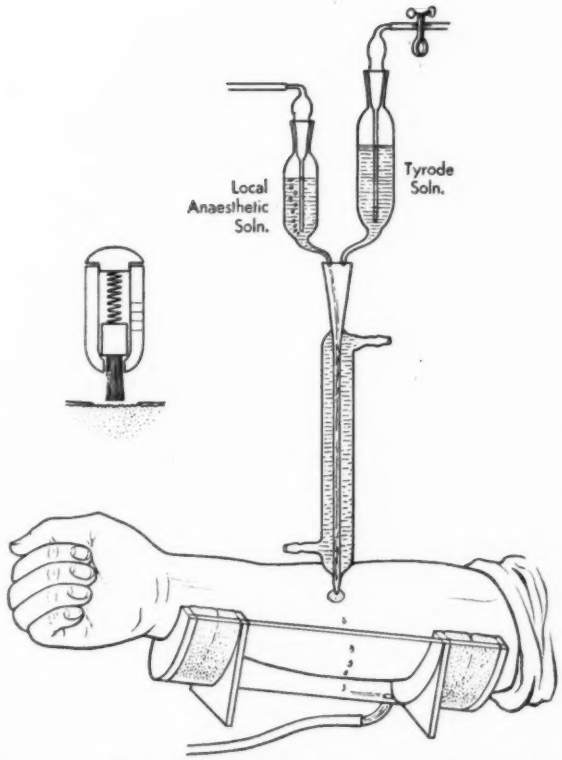


FIG. 1.—Arrangement of apparatus for applying local anaesthetic and Tyrode solutions to the area of exposed dermis. The inset shows the brush used for applying a stimulus to the area. For details, see text.

be moved once it has been brought into contact with the dermis, and must not touch the edge of the blister base; it should be dried between tests.

The degree of anaesthesia was assessed in terms of a three-point scale as slight, appreciable, and full anaesthesia (scored as 1, 2, and 3). The testing procedure consisted of two parts: calibration and assay.

For calibration full anaesthesia was first produced by applying a 0.2% procaine solution. (0.4% was sometimes needed on a freshly exposed area.) The degree of anaesthesia was such that after about 1 min. the standard stimulus could hardly be felt. This is a well-defined point on the scale, and it was usually necessary to determine it only once. Intermediate degrees of anaesthesia were produced by applying, repeatedly if the subject found it necessary, 0.025, 0.05, and 0.1% solutions of procaine. The standard stimulus was applied and the subject was asked to assess the response. Some guidance was given in allocating a score. Score 1 was allocated for a sensation in which the sharp pricking of the individual hairs of the baseline stimulus could no longer be distinguished, score 2 for one in which the edges of the brush could not be clearly felt but the stimulus was still easily perceived, and score 3 for full anaesthesia, in which the stimulus could only be detected by a slight perception of pressure.

The assay itself was carried out by a (2 + 2) design. It usually consisted of 20 "tests," each solution being tested 5 times. A test was carried out as follows. The local anaesthetic solution was allowed to flow over the exposed area; 30, 60, and 90 sec. after the test was begun the flow was interrupted and three stimuli were applied at intervals of about 2 sec. The anaesthetic solution was then replaced by Tyrode solution and a further 90 to 120 sec. were allowed for washing out the anaesthetic and checking the base line. The duration of a test cycle was thus 3 to 3½ min. Although it is desirable for the comfort of the subject to complete the testing within two hours, the area remained sensitive for many hours and was on one occasion used for a further assay on the second day. The assay was carried out as a "double blind" test in which neither subject nor tester knew which solution was used. In most assays the solutions were applied in randomized order in blocks of four, but in some instances a completely randomized design was adopted.

For the test on the exposed dermis the anaesthetic solutions were made up in Tyrode solution of *p*H 7.4. Tyrode solution is well buffered, and the presence of a local anaesthetic in a concentration below 0.1% did not alter its *p*H by more than 0.05 units.

*Intradermal Wheal in the Guinea-pig.*—These tests were carried out by a slight modification of the method of Bülbring and Wajda (1945), using four wheals (instead of two) on the back of each animal. In one experiment the intradermal wheal method in both the guinea-pig and in man was used in parallel assays. This experiment was carried out with solutions made up in M/5 phosphate at *p*H 7.0.

All drugs were used as the hydrochloride and all concentrations refer to the salt.

## RESULTS

### *1. Intradermal Wheal Method*

Fig. 2 shows a comparison of lignocaine and procaine on 52 subjects in a (2+2) assay with 0.1 and 0.4% lignocaine and 0.25 and 1.0% procaine solutions. Analysis of variance shows that the regression lines do not differ significantly from parallelism. The activity of lignocaine was calculated to be 2.6 times greater than that of procaine with limits of 2.2 to 3.1 for P=0.05.

Fig. 3 shows a comparison of cinchocaine and procaine on 46 subjects in a (3+3) assay; procaine 0.4, 1, and 2.5%; and cinchocaine 0.01, 0.025, and 0.062% was used. The detailed results are given in Table I. The mean scores were 14.5, 19.8, and 24.3 for the 3 solutions of procaine and 17.8, 21.0, and 26.4 for the 3 solutions of cinchocaine. There was no significant deviation of the regression lines from linearity or parallelism (Emmens, 1948). Cinchocaine was 49 times more active than procaine with limits of 39 and 61 for P=0.05. A further assay with only four test solutions (pro-

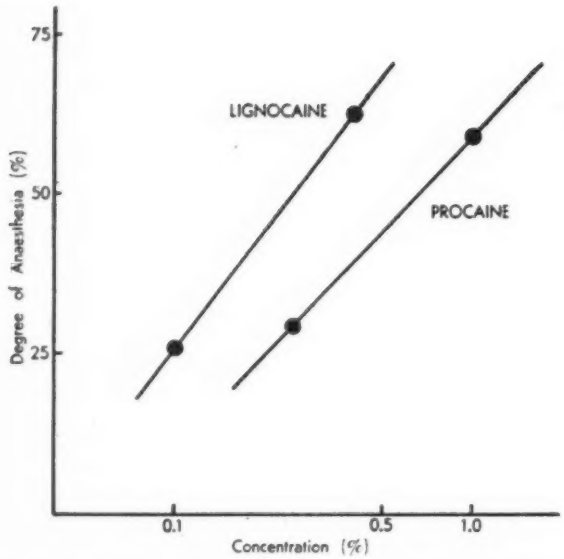


FIG. 2.—Mean results of a comparison of the activity of lignocaine and procaine on 52 subjects in a (2+2) assay using the intradermal wheal method; lignocaine 0.1% and 0.4%, procaine 0.25% and 1·0%. Lignocaine was 2.6 times more active than procaine with limits of 2.2 to 3.1 for P=0.05.

caine 0.5 and 2.5% ; cinchocaine 0.01 and 0.05%) on 57 different subjects gave an activity ratio of 50, with limits of 43 and 59 for P=0.05.

*Time Course of Anaesthesia.*—Fig. 4 shows the time course of anaesthesia with procaine and cinchocaine. The records were obtained by plotting against time the mean degree of anaesthesia for each of the six successive tests. The time course of anaesthesia varies with the drug used and its concentration. With 0.4% procaine full anaesthesia lasts for only a few minutes, whereas with 2.5% procaine anaesthesia is complete for 10–15 min. The rate at which anaesthesia wears off is independent of the initial concentration injected ; anaesthesia of the wheal injected with 2.5% procaine decreases just as quickly as anaesthesia of the wheal injected with 0.4% procaine. The rate of decrease of anaesthesia with cinchocaine is appreciably slower than with procaine.

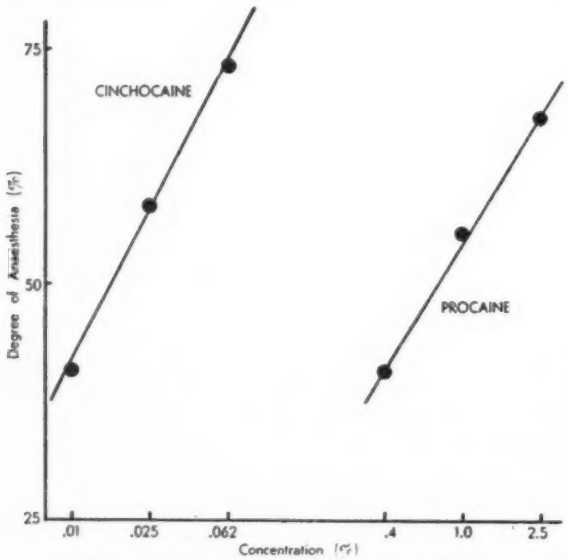


FIG. 3.—Mean results of a comparison of cinchocaine and procaine on 46 subjects in a (3+3) assay using the intradermal wheal method; cinchocaine 0.01%, 0.025%, and 0.062%, procaine 0.4%, 1.0%, and 2.5%. Cinchocaine was 49 times more active than procaine with limits of 39 to 61 for P=0.05.

TABLE I

(3+3) ASSAY OF CINCHOCAINE AGAINST PROCAINE: INDIVIDUAL RESPONSES OF 46 SUBJECTS TESTED IN 6 GROUPS

Response for total anaesthesia=36. The regression lines are linear and do not deviate significantly from parallelism. Cinchocaine is 49 times more active than procaine, with limits of 39 to 61 for P=0.05.

| Expt. No. | Procaine | | | Cinchocaine | | |
|---|---|---|---|---|---|---|
| | 0·4% | 1·0% | 2·5% | 0·01% | 0·025% | 0·062% |
| 1 | 22 | 23 | 22 | 22 | 22 | 26 |
| | 23 | 23 | 27 | 23 | 21 | 33 |
| | 18 | 18 | 23 | 17 | 23 | 30 |
| | 23 | 27 | 29 | 25 | 30 | 30 |
| | 14 | 21 | 22 | 8 | 19 | 24 |
| | 15 | 29 | 21 | 17 | 28 | 22 |
| | 12 | 28 | 30 | 16 | 36 | 32 |
| | 14 | 31 | 31 | 14 | 34 | 29 |
| 2 | 27 | 24 | 26 | 12 | 20 | 31 |
| | 11 | 28 | 20 | 19 | 11 | 34 |
| | 27 | 30 | 25 | 24 | 31 | 36 |
| | 22 | 22 | 24 | 27 | 28 | 24 |
| | 13 | 24 | 34 | 20 | 17 | 28 |
| | 12 | 17 | 26 | 14 | 24 | 32 |
| | 9 | 16 | 23 | 7 | 17 | 30 |
| | 13 | 24 | 31 | 19 | 21 | 32 |
| | 11 | 31 | 24 | 15 | 30 | 33 |
| 3 | 12 | 17 | 25 | 10 | 24 | 27 |
| | 8 | 20 | 17 | 10 | 15 | 26 |
| | 10 | 10 | 29 | 19 | 19 | 31 |
| | 20 | 20 | 33 | 17 | 22 | 27 |
| | 11 | 10 | 28 | 10 | 20 | 27 |
| | 10 | 11 | 19 | 11 | 16 | 27 |
| | 13 | 20 | 21 | 10 | 23 | 22 |
| | 16 | 20 | 33 | 20 | 21 | 30 |
| 4 | 11 | 18 | 19 | 8 | 16 | 22 |
| | 9 | 13 | 19 | 16 | 19 | 20 |
| | 12 | 21 | 25 | 13 | 22 | 24 |
| | 16 | 27 | 18 | 15 | 21 | 23 |
| | 14 | 18 | 26 | 9 | 22 | 30 |
| | 22 | 24 | 29 | 23 | 20 | 33 |
| 5 | 14 | 13 | 25 | 16 | 19 | 27 |
| | 11 | 16 | 25 | 16 | 19 | 26 |
| | 11 | 16 | 24 | 12 | 16 | 22 |
| | 12 | 17 | 19 | 6 | 18 | 8 |
| | 10 | 16 | 23 | 1 | 24 | 19 |
| | 11 | 17 | 29 | 12 | 20 | 28 |
| | 15 | 14 | 25 | 12 | 16 | 21 |
| 6 | 23 | 23 | 32 | 16 | 14 | 26 |
| | 12 | 12 | 28 | 16 | 12 | 28 |
| | 18 | 16 | 15 | 9 | 16 | 22 |
| | 10 | 20 | 21 | 12 | 16 | 20 |
| | 16 | 19 | 18 | 20 | 13 | 18 |
| | 17 | 13 | 21 | 14 | 26 | 26 |
| | 9 | 12 | 12 | 14 | 20 | 23 |
| | 10 | 23 | 22 | 16 | 27 | 23 |
| Means | 14·5 | 19·8 | 24·3 | 14·8 | 21·0 | 26·4 |

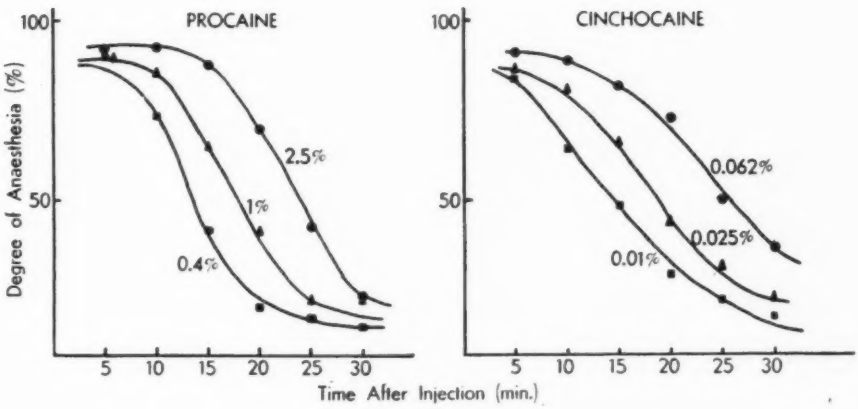


FIG. 4.—Time course of anaesthesia in intradermal wheals using 0.01%, 0.025%, and 0.062% cinchocaine, and 0.4%, 1.0%, and 2.5% procaine. The rate at which the anaesthesia wears off is slower with cinchocaine than with procaine.

The average time for a decline of anaesthesia from 80% to 20% is 14 min. for cinchocaine, and 10 min. for procaine.

*Comparison of Human and Guinea-pig Wheal Test.*—The value of 50 found for the activity of cinchocaine relative to procaine when tested on the human wheal is 2 to 5 times greater than published values for tests done on the guinea-pig wheal: Bülbring and Wajda (1945) found a value of about 23; Summers and Edge (1947), using a slightly modified version of the same method, found cinchocaine to be 24 times more active, whilst Elio (1948), using the original method, estimated the relative activity to be only 10. In unpublished class experiments with guinea-pigs we obtained an activity ratio of 21.

This species difference was confirmed by carrying out parallel assays in the guinea-pig and in man with solutions made up in M/5 phosphate buffer. Each assay consisted of 12 groups. The results are given in Table II. The presence of the phosphate buffer at *p*H 7.0 appeared to enhance the activity of the cinchocaine in both species, but the human result is again several times higher than that obtained on guinea-pigs.

TABLE II

COMPARISON OF GUINEA-PIG AND HUMAN WHEAL METHODS FOR ASSAY OF CINCHOCAINE AGAINST PROCAINE

Solutions in M/5 phosphate buffer at *p*H 7.0.

| Test Solution | Degree of Anaesthesia (out of 36). Each Result is the Mean of 12 Tests | |
|---|---|---|
| | Guinea-pig | Man |
| Procaine, 0·1% .. .. | 7·2 | — |
| ,, 0·5% .. .. | 23·3 | — |
| ,, 0·4% .. .. | — | 7·0 |
| ,, 2·0% .. .. | — | 21·4 |
| Cinchocaine, 0·01% .. | 20·2 | 16·2 |
| ,, 0·05% .. | 30·8 | 26·1 |
| Relative activity (cinchocaine/procaine) | 34 | 100 |
| Species difference (man/guinea-pig) .. | 2·9 | |

*II. Exposed Dermis Method*

Table III shows the responses obtained during a typical assay comparing cocaine solutions (0.005 and 0.02%) with procaine solutions (0.02 and 0.08%). The subject had previously been used for an experiment of this kind. The exposed area of dermis was about 0.3 sq. cm. Twenty tests (5 groups of 4) were done at 3 min. intervals. The responses elicited at 30, 60, and 90 sec. after the anaesthetic solution began to flow are given in detail to indicate the variation encountered. The test scores for the more concentrated solutions are about twice as great as those for a fourfold dilution; there is no overlap. Table IV shows the

TABLE III

DETAILS OF SCORES OBTAINED IN A TYPICAL ASSAY USING THE EXPOSED DERMIS METHOD

Each solution was tested five times. (Maximum score for each test = 27.)

S = 0·08% procaine. U = 0·02% cocaine.

| Group | Time (sec.) | U/4 | S/4 | U | S |
|---|---|---|---|---|---|
| 1 | 30 | 0 1 1 | 0 0 1 | 2 1 3 | 2 2 3 |
| | 60 | 1 1 1 | 1 2 2 | 2 2 2 | 2 2 2 |
| | 90 | 0 1 1 | 2 1 2 | 2 1 2 | 2 2 3 |
| 2 | 30 | 1 0 0 | 1 2 2 | 1 2 2 | 3 2 2 |
| | 60 | 1 1 2 | 1 1 1 | 2 2 2 | 3 3 3 |
| | 90 | 1 1 2 | 1 1 2 | 2 3 3 | 3 3 3 |
| 3 | 30 | 1 1 2 | 1 1 2 | 2 2 2 | 2 3 2 |
| | 60 | 1 1 2 | 2 2 3 | 2 3 2 | 3 3 3 |
| | 90 | 1 2 2 | 3 2 2 | 2 3 2 | 3 3 3 |
| 4 | 30 | 0 1 1 | 1 2 2 | 2 3 3 | 3 2 3 |
| | 60 | 0 0 1 | 2 3 2 | 2 3 2 | 3 3 3 |
| | 90 | 1 1 1 | 1 1 2 | 3 3 3 | 3 3 3 |
| 5 | 30 | 2 1 2 | 2 1 1 | 2 3 2 | 2 3 3 |
| | 60 | 1 2 1 | 2 1 2 | 2 3 2 | 3 3 2 |
| | 90 | 1 2 1 | 1 2 2 | 3 2 3 | 2 2 3 |

TABLE IV

TOTALS OF SCORES AND ANALYSIS OF VARIANCE OF A TYPICAL ASSAY USING THE EXPOSED DERMIS METHOD

(Maximum score for each test = 27.)

S = 0·08% procaine. U = 0·02% cocaine.

| Group | U/4 | S/4 | U | S | Total |
|---|---|---|---|---|---|
| 1 | 7 | 11 | 17 | 20 | 55 |
| 2 | 9 | 12 | 19 | 25 | 65 |
| 3 | 13 | 18 | 20 | 25 | 76 |
| 4 | 6 | 16 | 24 | 26 | 72 |
| 5 | 13 | 14 | 22 | 23 | 72 |
| | 48 | 71 | 102 | 119 | 340 |

| Variation | Sum of Squares | Degrees of Freedom | Mean Squares | P |
|---|---|---|---|---|
| Groups .. | 68 | 4 | 17 | < 0·01 |
| Anaesthetics | 80 | 1 | 80 | < 0·01 |
| Regression | 530 | 1 | 530 | < 0·01 |
| Parallelism .. | 2 | 1 | 2 | > 0·2 |
| Error .. | 30 | 12 | 2·5 | |

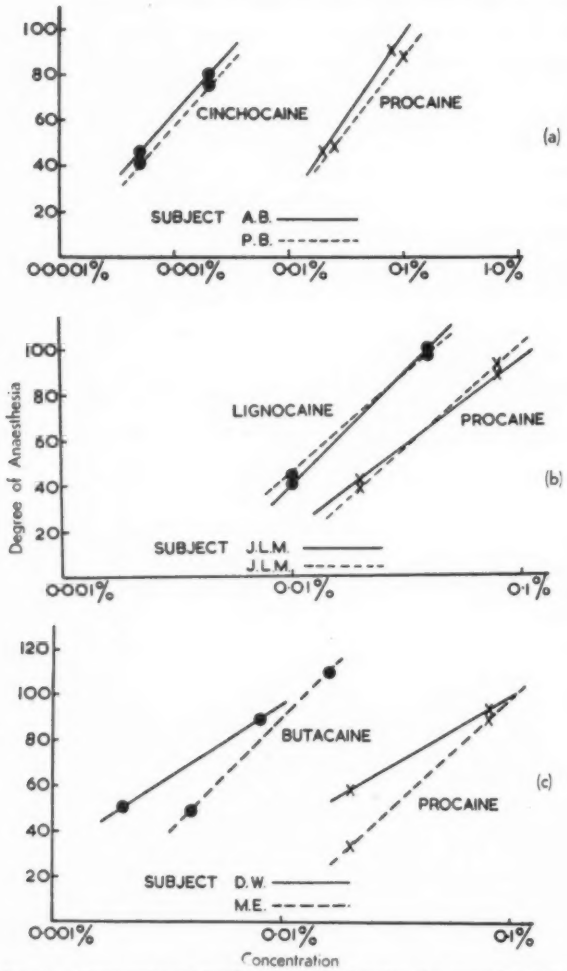


FIG. 5.—Three pairs of duplicate assays showing the reproducibility of assays done on the exposed dermis. The degree of anaesthesia is in terms of the test score. (Full anaesthesia = 135.)

total scores and the analysis of variance (Schild, 1942). The regression lines deviate significantly from parallelism and there is a highly significant difference between the scores for the two concentrations; the method is therefore valid for making quantitative comparisons. The accuracy of a comparison is surprisingly great in view of the fact that the subject had not been specially trained. By this test cocaine was 2.3 times more active (for infiltration anaesthesia) than procaine, with limits of error of 1.9 to 2.9 (P = 0.05).

The assays were reproducible. This is illustrated in Fig. 5, which shows three pairs of duplicate assays. One pair (Fig. 5b) was done on consecutive days on the same subject, using the same blister base. The other two pairs were done on four different subjects none of whom had been previously tested. The difference in slope of the regression lines on the two butacaine and procaine assays (Fig. 5c) reflects the difference of the assessment of the degree of anaesthesia. However, within each assay the regression lines for the two drugs were parallel.

Table V gives the relative activities of six local anaesthetics tested against procaine by the exposed dermis method. Each substance was tested twice; in five cases a different subject was used for the second test. Analysis of variance showed that there was no deviation from parallelism at the 1% level of significance.

TABLE V

ACTIVITIES OF LOCAL ANAESTHETICS RELATIVE TO PROCAINE MEASURED ON THE EXPOSED DERMIS OF THE FOREARM

| Anaesthetic | Relative Activity | | Limits of Error (P = 0·05) |
|---|---|---|---|
| | Duplicate Assays | Mean Result | |
| Cocaine .. .. | 2·31<br>2·67 | 2·5 | 1·9– 2·9<br>2·1– 4·4 |
| Cinchocaine .. | 33·0<br>32·6 | 32·8 | 27–41<br>26–41 |
| Lignocaine .. .. | 2·28<br>2·30 | 2·3 | 1·8– 3·4<br>1·3– 4·2 |
| Butacaine .. .. | 7·86<br>8·06 | 8·0 | 4·1–15·1<br>4·7–13·7 |
| Amethocaine .. | 27·4<br>41·6 | 34·5 | 20–37<br>19–68 |
| Ravocaine .. .. | 9·55<br>6·02 | 7·6 | 4·2–17·4<br>4·2– 8·6 |

*Duration of Test.*—The question arises whether the duration of contact of the local anaesthetic solution with the exposed area of dermis is a significant variable in the assessment of the relative activity of two local anaesthetics. It was found that with all local anaesthetics tested except cinchocaine a continuous flow of solution over the area produced a degree of anaesthesia in 30 sec., which was 80 to 90% of the average value for the whole

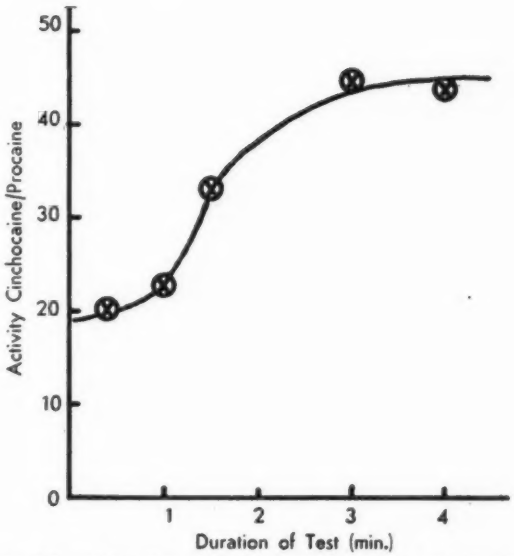


FIG. 6.—Effect of duration of test period on the activity of cinchocaine relative to procaine. The activity increases with time of contact for test periods of less than 3 min.

test. Cinchocaine is an exception ; it acts more slowly than procaine and the activity ratio of the two drugs depends on the duration of the test. This is shown in Fig. 6, which gives the results of five assays of varying duration. Cinchocaine is about 20 times more active than procaine when tested after a contact time of 30 sec. but 45 times more active when tested after 3 or 4 min. contact. After this time the activity ratio reaches a steady value. The activity ratio obtained with the standard contact time of 90 sec. is thus 27% less than the steady value. Lignocaine, butacaine, amethocaine and ravocaine also appeared to act slightly more slowly than procaine, and cocaine more rapidly ; but the differences are small compared with those shown by cinchocaine.

TABLE VI

PRECISION COEFFICIENTS OF ASSAYS

The value of $\lambda$ is equal to the ratio standard error/slope of regression line and is an estimate of the variability from test to test

| Substance | | Design | Precision Coefficient |
|---|---|---|---|
| Lignocaine | Human intradermal wheal (class experiment) | 2+2 | 0·36 |
| Cinchocaine | " " " | 3+3 | 0·40 |
| " | " " " | 2+2 | 0·27 |
| " | Human intradermal wheal (research) | 2+2 | 0·30 |
| " | Guinea-pig intradermal wheal (research) | 2+2 | 0·38 |
| Cocaine .. | Human exposed dermis | 2+2 | 0·092 and 0·120 |
| Cinchocaine | " " " | 2+2 | 0·092 " 0·102 |
| Lignocaine | " " " | 2+2 | 0·166 " 0·180 |
| Butacaine .. | " " " | 2+2 | 0·262 " 0·212 |
| Amethocaine | " " " | 2+2 | 0·136 " 0·240 |
| Ravocaine | " " " | 2+2 | 0·232 " 0·152 |

*Accuracy of Tests*

Table VI summarizes the precision of the various assays. Under class conditions the precision coefficient ($\lambda$) for assays by the intradermal wheal method in man averages 0.34 ; under research conditions it is 0.30. The corresponding value for the intradermal method with guinea-pigs is 0.38—i.e., the human subject is rather more discriminating than the guinea-pig. The coefficient for the exposed dermis method is consistently lower than for the intradermal wheal method. The average coefficient for the 12 assays in Table V is 0.155 (harmonic mean). This is about half the value of the intradermal wheal method. The exposed dermis method is thus considerably more precise: the same degree of accuracy can be obtained with one quarter the number of tests. A further advantage of the method is that many more tests can be done in one session. Five groups were usually done in one session as compared with only one group with the wheal method. In terms of numbers of subjects required for an assay of given accuracy the efficiency of the new method is therefore 20 times greater than that of the wheal method.

## DISCUSSION

The design of a test for comparing the activity of local anaesthetics appears to be a compromise between accuracy and relevance to conditions of use. Probably the most precise method and the simplest of interpretation is that which measures depression of conduction in an isolated nerve trunk. However, the results of such measurements do not relate to practical conditions of use ; factors such as diffusion to the nerve fibre and the buffering action of the surrounding tissue are ignored. On the other hand, *in vivo* tests on the intact animal are less accurate, but they do approximate to clinical conditions.

Despite the low precision of *in vivo* tests any desired accuracy can be obtained by increasing the number of tests. The three assays done by the intradermal wheal method on groups of about 50 subjects illustrate the degree of accuracy that can be achieved in this way. However, these assays are laborious and a method which gives similar accuracy with only 2 or 3 subjects is preferable.

The increase in precision obtained by using the exposed dermis method results from eliminating variations in the test area ; all the solutions can be tested repeatedly on the same site under identical conditions. In this respect the exposed dermis method approaches the conditions of an isolated

nerve preparation, and the question arises whether the results obtained by it are as relevant to clinical use as those obtained by the intradermal wheal method. Although the site of action is similar in both methods, diffusion and buffering of the tissue probably play a smaller part in the exposed dermis. The contact time in the exposed dermis method is much shorter than in the wheal method (or in clinical practice) and this may bias the results when the rate of action of the two substances is appreciably different. For example, cinchocaine had only 35 times the activity of procaine with a contact time of 90 sec., but with 3 min. contact the value has risen to 45, approximating that of 50 for the wheal. Thus, when testing local anaesthetics with differing rates of action, the exposed dermis test of 90 sec. is likely to bias the result slightly against the slower acting compound. The rates of action of the five remaining compounds tested (Table V) do not differ much from that of procaine, and the two methods would therefore be expected to agree. This was so for lignocaine, which was tested against procaine by both methods. It is surprising to find that lignocaine does not act more rapidly on the exposed dermis than procaine. This observation is in contrast to the claims made from observations in clinical trials (Gordh, 1949; Crawford, 1953).

It may be possible to increase the precision of the exposed dermis method by further subdividing the scale of anaesthesia. This would involve using trained subjects. We considered it undesirable to ask a subject to submit to frequent testing even though the blisters are small and heal readily. Nine of the assays in Table V have been done on subjects who were entirely new to the test; the remaining three assays were done on subjects who had been tested only once before. The use of untrained subjects for the test does not produce misleading results. If the limits of error of the estimate of relative activity are not sufficiently close, the test can be repeated and the results combined until the desired accuracy has been reached. On the other hand, tests done on the guinea-pig, however accurately performed, cannot be directly applied to man, since there is a systematic species difference between guinea-pig and man.


## Summary

1. Two methods for studying the activity of local anaesthetics in man are described. One is an intradermal wheal method. The other uses a small area of exposed dermis on the forearm (exposed dermis method).

2. Cinchocaine and lignocaine were compared with procaine by the wheal method. In a (3+3) assay with 46 subjects cinchocaine was 49 times more active than procaine (39 to 61, $P=0.05$); in a (2+2) assay with 57 subjects it was 50 times more active (43–59, $P=0.05$). In a (2+2) assay with 52 subjects lignocaine was 2.6 times more active than procaine (2.2–3.1, $P=0.05$).

3. By the intradermal method the activity ratio of cinchocaine and procaine was 2 to 3 times greater in man than in the guinea-pig.

4. By the exposed dermis method, activities relative to procaine were: cocaine 2.5, cinchocaine 33, lignocaine 2.3, butacaine 8.0, amethocaine 34, and ravocaine 7.6.

5. By both methods cinchocaine acted more slowly than procaine.

6. The precision of the intradermal wheal and the exposed dermis methods has been compared. The latter was about 20 times more efficient in terms of the number of subjects required for an assay of given accuracy.


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# PRESENCE OF ACETYLCHOLINE IN THE MALAYAN JACK-FRUIT, *ARTOCARPUS INTEGRA*

BY

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In higher animals acetylcholine (ACh) is associated with the transmission of nerve impulses. It has also been found in the free-living protozoon *Paramoecium* (Bayer and Wense, 1936), in the bacterium *Lactobacillus plantarum* (Stephenson and Rowatt, 1947), in the parasite *Trypanosoma rhodesiense* (Bülbring, Lourie, and Pardoe, 1949), and in the gill plates of the mussel *Mytilus edulis* (Bülbring, Burn, and Shelley, 1953). In these lower organisms there is no transmission of nerve impulses, but, as ACh is associated with such motor activity as ciliary movement, Bülbring *et al.* (1953) suggested that ACh might act in these organisms as a local hormone. In plants ACh has so far been found only in ergot, *Claviceps purpurea* (Ewins, 1914), and in the nettles *Urtica urens* and *Urtica diocia* (Emmelin and Feldberg, 1947, 1949). The present communication reports another rich plant source of ACh—the seed and leaf of the Malayan Jack-fruit, *Artocarpus integra*.

## METHODS

*The Jack-fruit and its Seed.*—The Jack-fruit is a compound fruit borne by a tree, *Artocarpus integra* (Fig. 1), belonging to a genus of some forty trees of the family *Urticaceae* found in South-east Asia. In Malay it is called "Nangka." According to Burkill (1935), it was originally found in India and then brought to Malaysia. The Jack-fruit tree is perennial and may bear fruits at any time of the year. The fruit, generally oblong, usually grows to an enormous size (Fig. 2A) and weighs about 40 lb. The external surface is like a beehive and is greenish-yellow. One Jack-fruit may contain forty to sixty small, fleshy, yellow units of edible fruit (Fig. 2B) with a mawkishly sweet and "mousy" taste, each of which has a prominent seed (Fig. 2C) weighing 5–10 g. The seeds are usually discarded, but after being boiled with salt they can be eaten as a vegetable. The seeds have two covering layers. The thicker external layer is brown when fresh but grey when dry; it is attached loosely to the inner layer, which is fawn-coloured and attached rather closely to the seed tissue, from which it can be removed only by scraping with a knife. The flesh of the fruit is claimed to be laxative, irritating, and indigestible when it is unripe. Nursing mothers are not supposed to eat it in Malaya, as it is stated to lead to secretion of a deleterious substance in the milk.

The Jack-fruit seeds used in the present experiments were from ripe fruits, except when specific correlations were attempted between the ACh content of the seed and the ripeness of the fruits. The seeds were separated from the flesh, washed with tap water, and used fresh

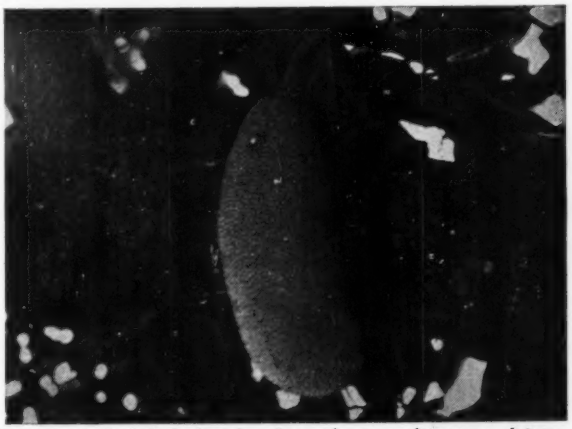

FIG. 1.—A Jack-fruit hanging from the tree *Artocarpus integra*.

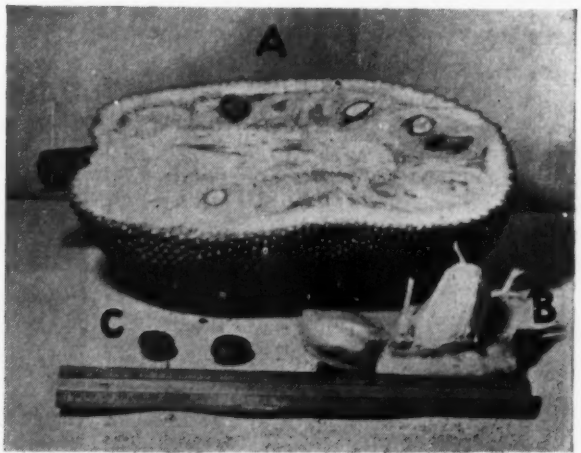


FIG. 2.—A, Longitudinal section of a Jack-fruit. B, Two small units of the fruit. C, Two seeds of the fruit.

or after storing in a refrigerator. In some experiments fresh leaves and newly cut stems of the fruit were also examined.

*Preparation of the Seed Extract.*—The two covering layers were removed. The seeds were immediately weighed, crushed in a mortar, and then macerated with acidified distilled water (1 ml. of N/3 HCl to 9 ml. of distilled water for each g. of seed), until the entire seed tissue was finely divided into granules. The supernatant fluid of the extract was centrifuged off and transferred into a graduated cylinder to which more acidified distilled water was added until the volume corresponded to 10 ml. for every g. of the peeled seed. The *p*H of this extract was about 3. For most of the pharmacological tests such a concentration of the extract was too high ; it was reduced to 1 to 2 mg./ml. by diluting it before use with Ringer, Locke or Tyrode solution. This also brought the *p*H of the extract to a level suitable for testing on the heart and other isolated tissues.

*Pharmacological Tests.*—The extracts were tested on the following conventional preparations : (1) on the guinea-pig ileum suspended in a bath of 8 ml. filled with Tyrode solution ; (2) on the virgin rat uterus suspended in a bath of 8 ml. filled with Locke solution containing only one-quarter of the normal amount of calcium chloride ; in both preparations the physiological solutions were aerated with a 95% $O_2$ and 5% $CO_2$ mixture and maintained at 37.5° C. ; (3) on the isolated toad heart perfused with Ringer solution through a Symes cannula ; (4) on the isolated toad rectus abdominis muscle suspended in a bath of 6 ml. containing eserinized Ringer solution at room temperature (about 30° C.) and aerated with air ; and (5) on the arterial blood pressure of cats anaesthetized with chloralose.

*Estimation of Cholinesterase Activity and Chemical Precipitation of ACh.*—Estimations of cholinesterase activity were made manometrically (Ammon, 1933 ; Augustinsson, 1948), or by incubating the seed extract at 37.5° C. in a bicarbonate Ringer solution containing ACh with and without eserine, and assaying the remaining ACh at the end of incubation on the toad rectus abdominis.

Chemical precipitation of the ACh from the seed extract was carried out according to the methods used by Dudley (1933), modified by Bülbring *et al.* (1953), to the step when the excess of barium was removed by adding sodium sulphate. The solution was then concentrated *in vacuo* and adjusted to *p*H 4 with HCl before it was assayed on the toad rectus for ACh. The standard acetylcholine chloride was freshly prepared each time from a stock solution (Hoffmann-La Roche glass ampoules).

## Results

### *Pharmacological Actions of the Jack-fruit Seed*

*Isolated Guinea-pig Ileum.*—Fig. 3A shows that the seeds contained a substance strongly stimulating the smooth muscle. The contraction caused by 0.25 mg. of the seed was greater than that produced by 0.1 μg. ACh. This stimulating action was lost when the seed extract was treated with NaOH (Fig. 3A, d and e). In an acid medium the stimulating substance was, however, very stable. When tested on an atropinized preparation, the stimulating action of the seed disappeared, even when the seed extract was added in a large dose (Fig. 3B, h).

*Isolated Toad Rectus Abdominis.*—Fig. 4A shows that 1 mg. of the seed caused a contraction of the muscle (at b) slightly bigger than that due to 0.5 μg. ACh (at a). The stimulating action of the seed was lost after the seed extract had been treated with NaOH. Further, (+)-tubocurarine added to the perfusion fluid rendered the muscle insensitive to ACh as well as to the seed extract (Fig. 4B). In concentrations of 1 and 5 μg./ml. of Ringer solution tubocurarine caused an identical degree of inhibition to the stimulating actions of 0.5 μg. ACh and 1 mg. of Jack-fruit seed.

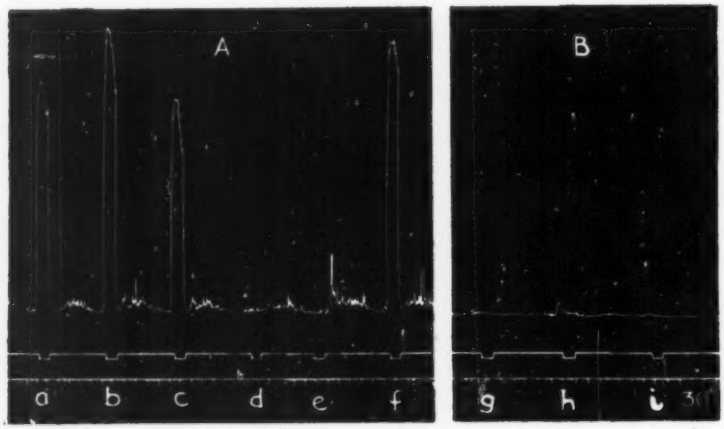


FIG. 3.—Contractions of guinea-pig ileum on the addition of the following substances to the bath: a, c, and f, 0.1 μg., and g, 2 μg. ACh; b, 0.25 mg., and h, 10 mg. of Jack-fruit seed; d, 0.25 mg., e, 20 mg., and i, 100 mg. of Jack-fruit seed treated with NaOH. Between A and B atropine sulphate $1.5 \times 10^{-6}$ added to the bath.

Extracts of the flesh of the fruit caused no contraction of the rectus muscle.

The stimulating substance in the Jack-fruit seeds and in the leaves appears to be present in a freely soluble form, for when a thin slice of the seed or a small shred of the leaf was dropped into the toad's rectus muscle bath the muscle began to contract in about 5–10 sec.

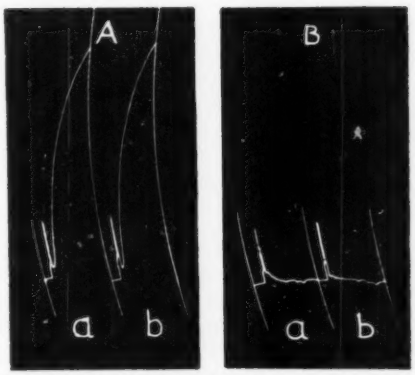


FIG. 4.—Contractions of toad rectus abdominis produced by 0.5 μg. ACh at a and by 1 mg. of Jack-fruit seed at b. Between A and B, 5 μg./ml. (+)-tubocurarine added to the bath.

*Isolated Toad Heart.*—Fig. 5 shows that on the isolated toad heart 0.4 mg. of the seed had a strong inhibitory effect which was abolished by atropine (Fig. 5A and B). When the seed extract was boiled with NaOH and then neutralized, it no longer exerted a cardio-inhibitory effect (Fig. 6B). On the contrary, it then had a stimulating action (Fig. 6B). This effect was also demonstrable with the untreated seed extract when tested on the atropinized heart. Extract corresponding to 2 mg. of the seed, which was five times bigger than that which had caused cardiac arrest before atropine (Fig. 5A), when injected into the Symes cannula after atropine caused strong, long-lasting stimulation of the heart (Fig. 5B). The Jack-fruit seed thus contains, in addition to the ACh-like substance, another pharmacologically active substance which has a stimulating action on the toad heart; its effect is normally masked by that of the ACh-like substance. The stimulating substance is also partly destroyed by the brief boiling in alkaline medium, because 50 mg. of the boiled seed was required to produce a stimulating effect (Fig. 6B), whereas 2 mg. of untreated seed produced a stronger response on the atropinized preparation (Fig. 5B). Repeated attempts to demonstrate the stimulating action of the Jack-fruit seed on the isolated perfused rabbit heart failed.

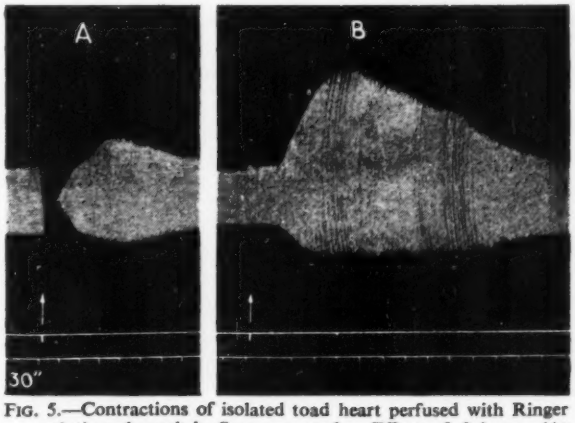


FIG. 5.—Contractions of isolated toad heart perfused with Ringer solution through a Symes cannula. Effect of 0.4 mg. (A) and of 2 mg. (B) of Jack-fruit seed. Between A and B 2 μg./ml. atropine sulphate added to the solution.

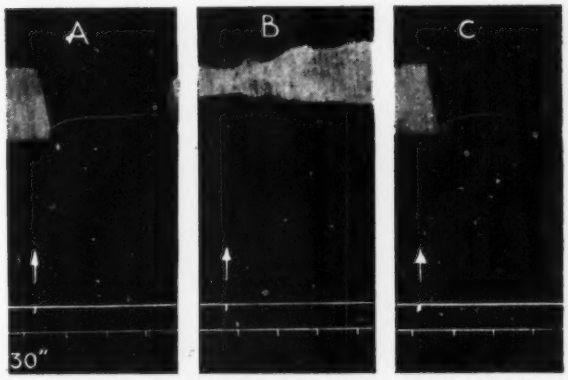


FIG. 6.—Contractions of isolated toad heart perfused with Ringer solution through a Symes cannula. A shows effect of 0.4 mg. Jack-fruit seed, B of 50 mg. NaOH-treated Jack-fruit seed, and C of 0.3 μg. ACh.

*Cat's Arterial Blood Pressure.*—Fig. 7A shows that the intravenous injection of extract corresponding to 0.5 mg. of the Jack-fruit seed caused a fall in the arterial blood pressure similar to that produced by 0.2 μg. ACh. This depressor effect was increased about fivefold after eserine (0.25 mg. intravenously) and abolished by atropine (Fig. 7B). An extract of the Jack-fruit seed treated with alkali no longer lowered the blood pressure, even when 50 mg. was injected. There was no indication of a stimulating action on the cat heart, either after atropine or when large doses of alkali-treated extract were injected.

*Isolated Virgin Rat Uterus.*—Fig. 8 shows that extract corresponding to 1.6 mg. of the Jack-fruit seed (b) caused contraction of the uterus almost exactly equal to that caused by 0.4 μg. ACh (a). As expected, 10 μg. of histamine caused slight relaxation of the rat uterus (Fig. 8c), whereas a thin slice of unextracted Jack-fruit seed added to the

bath produced strong contraction (Fig. 8d). These results, together with the observation made on the atropinized guinea-pig ileum, indicate that no histamine is present in the Jack-fruit seed.

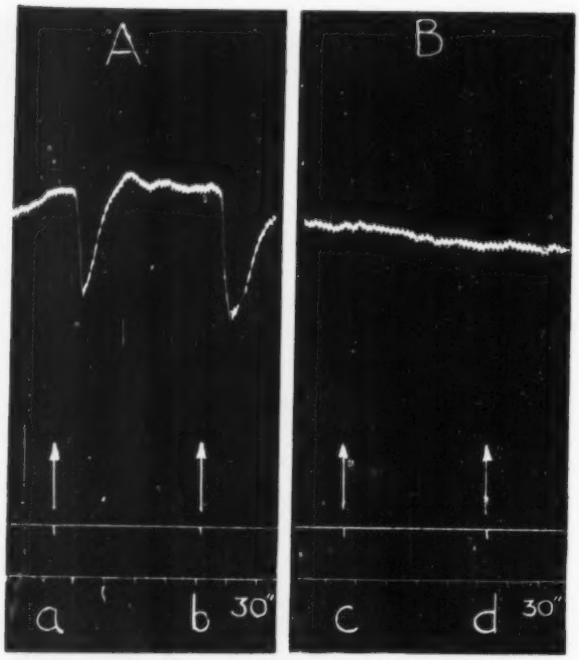


FIG. 7.—Arterial blood pressure of cat anaesthetized with chloralose. Intravenous injections of 0.5 mg. and of 2 mg. of Jack-fruit seed at a and d, and of 0.2 $\mu$g. and 1 $\mu$g. ACh at b and c. Between A and B, 2 mg. atropine sulphate injected intravenously.

*Assay of Jack-fruit Seed Extracts by Different Methods.*—It is generally accepted that, if the unknown substance in an extract gives the same value when assayed against the same standard solution by different biological preparations, it can be taken as evidence that the unknown substance is the same as the standard. Three separate extracts of Jack-fruit seeds were therefore prepared and tested against a standard ACh solution on various preparations. The values obtained are shown in Table I, and they can be said to be identical for each method within the limit of experimental error. This strongly supports the claim that the depressor and muscle stimulating substance in the Jack-fruit seed is ACh. The activity will therefore be referred to as being due to ACh.

TABLE I

ACETYLCHOLINE CONTENT OF JACK-FRUIT SEED ASSAYED BY DIFFERENT METHODS

| Extract No. | Preparation | ACh Content in $\mu$g./g. |
|---|---|---|
| 1 | Cat B.P. .. .. .. | 350 |
| | Toad rectus .. .. | 310 |
| | „ heart .. .. | 350 |
| 2 | Toad rectus .. .. | 430 |
| | Guinea-pig ileum .. | 440 |
| 3 | Toad rectus .. .. | 500 |
| | Guinea-pig ileum .. | 550 |

*Precipitation of ACh from Jack-fruit Seed Extract*

Eight Jack-fruit seeds with a total weight of 48.2 g. were crushed in a glass mortar into small pieces and macerated with acidified water until they had been converted to a pulp-like mixture. The extract was then squeezed through cheese-cloth, to remove the insoluble material, and the filtrate was centrifuged twice to remove sediments. One ml. of the supernatant fluid was diluted with acidified Ringer's solution (0.4 ml. N/3 HCl to 100 ml. of Ringer's solution) and, when assayed for ACh on a toad rectus abdominis muscle, it gave a value of 720 $\mu$g./g. From this value the total amount of ACh present in the seed extract was computed. The results of the two experiments in which the yields of ACh precipitated from the seed extract together with that precipitated from a standard ACh solution are presented in Table II. It shows that an appreciable amount of ACh could be recovered by precipitation from the Jack-fruit seed extract. The yield values of 38.3% and 27.9% appear to be rather low, but the substance precipitated was undoubtedly ACh, as shown by its pharmacological actions and alkaline instability.

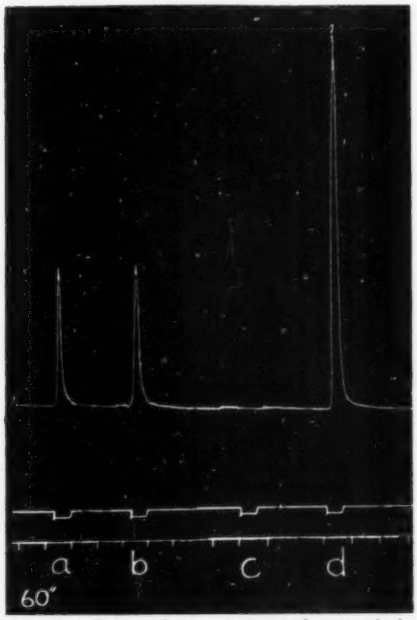


FIG. 8.—Contractions of isolated rat virgin uterus. At a effect of 0.4 $\mu$g. ACh, at b of 1.6 mg. Jack-fruit seed, at c of 10 $\mu$g. histamine, and at d of a thin slice of Jack-fruit seed dropped into the bath.

TABLE II

RESULTS OF CHEMICAL PRECIPITATION OF ACETYLCHOLINE FROM JACK-FRUIT SEEDS

| Expt. | Amount of Jack-fruit Seed Used | ACh Content μg./g. By Assay | Total ACh | ACh Recovered By Assay | Yield |
|---|---|---|---|---|---|
| 1 | — | — | Std. ACh 5·0 mg. | 2·375 mg. | 47·5% |
| 2 | 6·52 g. (one seed) | 450 | 2·95 mg. | 1·120 mg. | 38·3% |
| 3 | 48·20 g. (six seeds) | 720 | 30·70 mg. | 8·58 mg. | 27·9% |

*ACh Content of the Jack-fruit Seed, Leaf, and Stem*

The amounts of the active substance present in the seed and other tissues of the Jack-fruit tree were assayed against a standard ACh chloride solution on the isolated toad rectus abdominis. Forty-one estimations were made on the seeds of ripe and unripe fruit collected during a period of nine months, and the results obtained gave ACh content values varying from 300 to 1,000 μg./g. of fresh seed, with an average value of 564 μg./g.

A comparison was made of the extraction of ACh from seeds by acidified distilled water, 5% trichloracetic acid and 95% ethyl alcohol, but a higher value of ACh was always obtained from the seeds with acidified distilled water than with the other two solvents. There was no correlation between the ACh content and the size of the Jack-fruit seed or the ripeness of the fruits. The ACh content of the seeds was not affected by storage for a period of one month or more in the refrigerator. Drying of the seeds, however, resulted in a rapid fall of the ACh content. For example, the average ACh content of three seeds from the same fruit was 600 μg./g. when fresh and 100 μg./g. after being dried in a container surrounded by hot water for 72 hr.

The leaves of the Jack-fruit tree had an average water content of 60%, and five separate determinations gave an average ACh value of 60 μg./g. (1.6–120 μg./g.). On two occasions the midribs of several Jack-fruit leaves combined were extracted and found to have an ACh content of 250 and 350 μg./g. respectively. A short length of woody stem bearing the leaves was found to contain 8 μg./g. of ACh.

The seeds of another fruit, borne by a tree closely related to the Jack-fruit tree called *Artocarpus champeden* (or "Chempedak" in Malay), contained an average amount of only 3.0 μg./g. of ACh in eight different estimations.

*Absence of Cholinesterase in the Jack-fruit Seed*

In six experiments Jack-fruit seed extract was incubated with ACh as follows: 2 ml. of a concentrated seed extract (250 mg./ml.) together with 1 ml. of a 0.4% ACh solution plus 1 ml. of bicarbonate Ringer solution were incubated in a small conical flask on a water bath at 37.5° C. and shaken at a rate of 75/min. Both the seed extract and the ACh solution were prepared in a bicarbonate Ringer solution so that the *p*H of the medium was maintained at 7.4. Usually, two sets of such duplicated samples were set up—one with 0.1 ml. of a 0.5% solution of eserine salicylate and the other without eserine. In addition, one set of duplicated samples was set up, in which the enzyme preparation had been boiled. At the end of 1 hr. incubation 2 ml. of N/3 HCl was added to each sample, which was then boiled in order to stop any enzyme action. The amount of ACh remaining in each sample was assayed on the toad rectus abdominis preparation. Out of six experiments carried out in this manner, two indicated some enzymic hydrolysis of the added ACh, but the other four showed no sign of cholinesterase activity. Similar experiments carried out with a rat brain extract as the source of the enzyme, however, always gave positive results.

Since these results were not conclusive, the more precise manometric method of estimating cholinesterase activity, by measuring the rate of $CO_2$ release from a bicarbonate Ringer solution in a Warburg microrespirometer, was employed. In these experiments the reaction mixture in each manometer vessel had a total volume of 2.1 ml., consisting of 1.6 ml. of a 0.4% ACh solution in the main compartment and 0.4 ml. of the Jack-fruit seed extract plus 0.1 ml. of bicarbonate Ringer solution in the side arm. When the effect of eserine on the cholinesterase activity was to be studied, the bicarbonate Ringer was replaced by the same volume of a 0.5% eserine salicylate solution. The vessels and manometers were filled with a 95% $N_2$ and 5% $CO_2$ mixture and constantly shaken in a water bath at 37.5° C. at a rate of 90/min. When a simultaneous test was carried out with the Jack-fruit seed extract and a rat brain extract, the vessels containing the brain extract gave an average net manometer reading increase of 24.5 mm./hr., whereas the two vessels containing Jack-fruit seed extract gave an average value of only 3 mm./hr., which was due entirely to non-enzymic hydrolysis of ACh.

In order to test if the ACh in the Jack-fruit seed could be hydrolysed enzymically by cholinesterase from another source, such as rat brain, six samples, each containing 1.6 ml. of a concentrated Jack-fruit seed extract (400 mg./ml.) in a manometer vessel, were set up and incubated as above. To

two of these samples, which served as control, 0.5 ml. of bicarbonate Ringer solution was added. To each of the other four, 0.4 ml. of rat brain extract was added, and 0.1 ml. of a 0.5% eserine salicylate solution was further added to the last two samples which contained both Jack-fruit seed and brain extracts. An example of the results obtained is shown in Table III.

TABLE III

MANOMETER READINGS SHOWING HYDROLYSIS OF THE ACETYLCHOLINE IN THE JACK-FRUIT SEED BY THE CHOLINESTERASE FROM RAT BRAIN

| Manometers No. .. | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Jack-fruit seed extract, 1·6 mg. .. .. | + | + | + | + | + | + |
| Bicarbonate Ringer in ml. | 0·5 | 0·5 | 0·1 | 0·1 | 0 | 0 |
| Rat brain extract, 0·4 ml. | 0 | 0 | + | + | + | + |
| Eserine salicylate, 0·1 ml. | 0 | 0 | 0 | 0 | + | + |
| Manometer readings: net increase in mm./hr. at 37·5° C. .. .. | 1·5 | 3·0 | 13·4 | 14·0 | 1·0 | 2·0 |

The readings of manometers 1 and 2, the vessels of which contained no rat brain, were practically the same as those of manometers 5 and 6, in the vessels of which the cholinesterase from rat brain was inhibited by eserine. This means that hydrolysis of ACh in the Jack-fruit seed was not the result of enzymic action. The readings of manometers 3 and 4, however, gave an average increase of 13.7 mm./hr., which showed that the Jack-fruit seed contained a substance which could be hydrolysed enzymically by rat brain cholinesterase but not if this enzyme was inhibited by eserine as in manometers 5 and 6. Besides demonstrating the absence of cholinesterase in the Jack-fruit seed, this further supports the conclusion that the main active substance in Jack-fruit is ACh.

## DISCUSSION

With pharmacological methods it could be shown that the seeds and leaves of the Jack-fruit tree, *Artocarpus integra*, contain considerable amounts of ACh. This finding was substantiated by the fact that the ACh in the seed could be chemically precipitated and hydrolysed by cholinesterase from rat brain.

The high ACh equivalent of over 500 μg./g. in the seed and of 60 μg./g. in the leaves of the Jack-fruit is surprising. Such high values of ACh are not found in animal tissues, but an even higher concentration was found in the nettle hair fluid (Emmelin *et al.*, 1947). Since the total weight of the leaves of one Jack-fruit tree may amount to more than 250 kg., the leaves alone must contain about 15 g. of ACh. Another 5 g. of ACh may be estimated to be present in the seeds, making a total of 20 g. in one tree. When taken by mouth, the seeds would probably be pharmacologically active; and this may be the reason why the seeds are only eaten after they are cooked with salt—a procedure which would hydrolyse the ACh.

The Jack-fruit seed differs from the nettle plant in three respects (Emmelin *et al.*, 1947): (1) it contains no histamine, which the nettle plant contains in considerable quantity; (2) it contains, besides ACh, no other smooth-muscle-stimulating substance, whereas the nettle plant does; (3) it contains a toad-heart-stimulating substance, the nature of which is as yet unknown. It seems to be fairly stable in alkaline medium, but the fact that it lacks a general stimulating action on hearts of higher animals speaks against it being a sympathomimetic amine or a cardiac glycoside.

The absence of cholinesterase in the Jack-fruit seed is perhaps not surprising, because, if it were present, such great quantities of ACh could not be stored there. Cholinesterase is also absent in the nettle plant (Emmelin *et al.*, 1947). It must be recalled that in the animal kingdom, where ACh transmits nerve impulses, its release is intermittent and cholinesterase is indispensable to ensure that the ACh acts only for very brief periods. Even in the lower organisms, the need for cholinesterase to prevent the accumulation of ACh in the vicinity of the cilia is still apparent (Bülbring *et al.*, 1953). In plants the function of ACh is still not clear. But in the nettle plant it might serve a defence purpose by causing a burning pain in the skin in association with histamine (Emmelin *et al.*, 1947). The function of ACh in the Jack-fruit seed is at present unknown. Obviously the ACh in the seed is the result of a slow process of synthesis and accumulation; probably the seeds of the Jack-fruit form but a depot of ACh synthesized somewhere else in the plant. This raises the question: where is the ACh formed, in the leaves or in the root? It also suggests that the Jack-fruit tree may offer excellent material for studying the biological synthesis of ACh in plants.

## SUMMARY

1. Pharmacological tests, comparative assays and chemical precipitation have established that the seed and the leaves of the Jack-fruit tree, *Artocarpus integra*, contain considerable amounts of acetylcholine (ACh).

2. The average values of ACh were 564 μg./g. of seed, 300 μg./g. of midribs of leaves and 60 μg./g. of whole leaves.

3. In contrast to the high ACh content of Jack-fruit seed, the seed of the fruit of a closely allied species, *Artocarpus champeden*, contained only 3 $\mu$g. ACh/g.

4. A heat- and alkali-stable toad-heart-stimulating substance was also found in Jack-fruit seed.

5. No cholinesterase activity was found in Jack-fruit seed.

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# RELEASE OF HISTAMINE BY A SUBSTITUTED BUTYLAMINE (L 1935): COMPARISON WITH COMPOUND 48/80

BY

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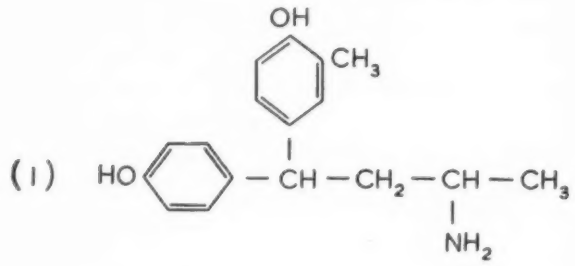
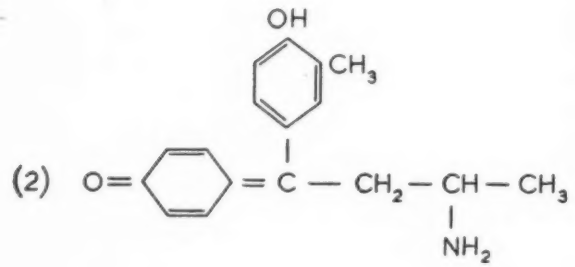
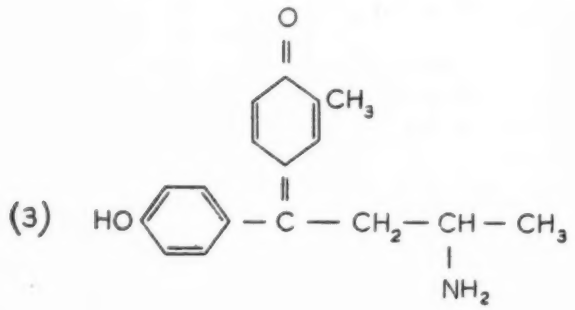
Charlier and his co-workers (Charlier, 1954; Charlier, Dallemagne and Philippot, 1954; Charlier and Vandersmissen, 1954) recently described the pharmacological properties of a substituted butylamine or methylpropylamine which is an equimolecular mixture of 3-4′-hydroxyphenyl-3-4″-hydroxy-3″-methylphenyl-1-methylpropylamine and its dehydro- form. The structural formula is given below. The substance consists of (1) with a tautomeric mixture of (2) and (3).







This substance, referred to in brief as L 1935, when injected intravenously into cats or dogs in doses of 0.25–1 mg./kg., caused a long-lasting fall of arterial blood pressure. Since the effect was prevented by promethazine, the authors suggested that it was accounted for, or at least partly, by release of histamine and that L 1935 was a histamine liberator. In man, an intravenous injection of 0.5 mg./kg. led to erythema and an urticarial rash with intense itching and some pain sensation, oedema of the face, and a fall in arterial blood pressure. With smaller doses (0.1 mg./kg.) erythema and mild itching only occurred (Lecomte, 1955). These findings also suggested a strong histamine-releasing property of L 1935. So far, however, no direct evidence for its histamine-releasing property has been brought forward.

The present experiments were undertaken to obtain this direct evidence by determining the release of histamine from perfused tissues and to compare the effect of L 1935 with that of the potent histamine liberator 48/80. In addition, a few experiments were performed on the arterial blood pressure of the cat in order to see if the depressor action of L 1935 showed the latency characteristic of histamine liberators. Finally, the effect of L 1935 was studied on the guinea-pig's ileum and compared with that of 48/80.

## METHODS

The histamine-releasing property was tested on perfused skin flaps of the cat's hind legs and on perfused hind quarters of the rat. The cats were anaesthetized with chloralose intravenously and the skin flaps perfused from the saphenous artery with Locke solution and the venous outflow collected from the saphenous vein, according to the method described by Feldberg and Paton (1951) and Feldberg and Schachter (1952). The rats were anaesthetized with pentobarbitone sodium intraperitoneally. The hind quarters were perfused from the lower abdominal aorta and the venous outflow collected from the vena cava, as described by Feldberg and Mongar (1954). In both

series of perfusion experiments the Locke solution contained 2 mg./ml. glucose. The venous effluent was assayed for histamine on the atropinized guinea-pig's ileum preparation. All values of histamine refer to the base.

For comparison of the histamine-releasing potency of L 1935 with that of 48/80 three injections were given in each preparation. The interval between injections was about 30 min. Either an injection of L 1935 was bracketed between two injections of 48/80, or an injection of 48/80 was bracketed between two injections of L 1935. The doses of either L 1935 or 48/80 used for the first and third bracketing injection were the same in each given preparation. This procedure was adopted because the amounts released by the same dose of a histamine liberator usually decrease with successive injections except when the amounts released are very small—for example, about 1 μg. histamine or less. For the assay it is best to adjust the dose used for injection so that it releases not more than a few micrograms of histamine, and that the output practically comes to an end within 30 min. In the experiments on the cat's skin, the flap from the left leg was always perfused first and the flap from one side was used to bracket L 1935, that from the other side to bracket 48/80. This made it possible to compare the results obtained from the two sides, particularly when the doses injected into both skin flaps were the same.

The cats whose skin flaps were perfused were kept under chloralose anaesthesia after the flaps had been removed, and were later used for comparing the effects of L 1935 and of 48/80 on the arterial blood pressure. For this purpose the left carotid artery was cannulated for recording the arterial blood pressure, and the right jugular vein for injections. The vagi were cut in the neck.

For the experiments on the isolated guinea-pig's ileum preparation a piece of ileum about 4 cm. long was suspended in 15 ml. magnesium-free Tyrode solution at 33° C.

## Results

### *Perfused Skin Flaps of Cat's Hind Legs*

L 1935 was found to be a potent histamine liberator when injected into the saphenous artery of the perfused skin flap of the cat's hind leg.

TABLE I

HISTAMINE RELEASE BY L 1935 FROM PERFUSED SKIN FLAPS OF THE CAT'S HIND LEGS

| L 1935 (μg. Injected) | Output of Histamine (μg.) |
|---|---|
| 0·5* | 4·03 |
| 1·0 | 8·50 |
| 2·0 | 2·34 |
| 2·0* | 3·52 |
| 2·4* | 12·3 |
| 2·5 | 14·9 |
| 2·5* | 1·74 |
| 2·5* | 9·29 |

* Injected 30 min. after an injection of 48/80.

TABLE II

COMPARISON OF THE HISTAMINE OUTPUT BY L 1935 AND COMPOUND 48/80 FROM PERFUSED SKIN FLAPS OF THE CAT'S HIND LEGS

| Expt. No. | Left Side | | Right Side | |
|---|---|---|---|---|
| | Injections of 48/80 or L 1935 (μg.) | Output of Histamine (μg.) | Injections of 48/80 or L 1935 (μg.) | Output of Histamine (μg.) |
| 1 | 0·5 48/80 | 11·6 | 1 L 1935 | 8·5 |
| | 0·5 L 1935 | 4·03 | 0·5 48/80 | 11·3 |
| | 0·5 48/80 | 4·99 | | |
| 2 | 2·5 L 1935 | 15·0 | 0·5 48/80 | 32·0 |
| | 0·5 48/80 | 17·9 | 2·5 L 1935 | 9·29 |
| | 2·5 L 1935 | 3·08 | 0·5 48/80 | 6·01 |
| 3 | | | 0·125 48/80 | 0·93 |
| | | | 2·5 L 1935 | 1·74 |
| | | | 0·125 48/80 | 0·68 |
| 4 | 0·125 48/80 | 0·17 | 2 L 1935 | 2·34 |
| | 2·0 L 1935 | 3·52 | 0·25 48/80 | 3·63 |
| | 0·125 48/80 | 0·75 | 2·0 L 1935 | 0·31 |
| 5 | 0·2 48/80 | 13·9 | 2·4 L 1935 | —* |
| | 2·4 L 1935 | 12·3 | 0·2 48/80 | 14·5 |
| | 0·2 48/80 | 4·14 | 2·4 L 1935 | 4·29 |

* Injection spoiled.

As seen from the results of Table I, the injection of 0.5–2.5 μg. resulted in the release of several μg. of histamine. When the histamine-releasing power of L 1935 was compared with that of 48/80, it was found that 48/80 was more potent. This is evident from the results of the five experiments given in Table II. From the results of experiments Nos. 1, 2, and 3 it is evident that 0.5 μg. 48/80 releases more histamine than do 0.5 μg., 1 μg., and 2.5 μg. of L 1935; that 2.5 μg. of L 1935, however, releases more histamine than does 0.125 μg. 48/80. Thus, 48/80 is more than five and less than twenty times as active as L 1935; the experiment

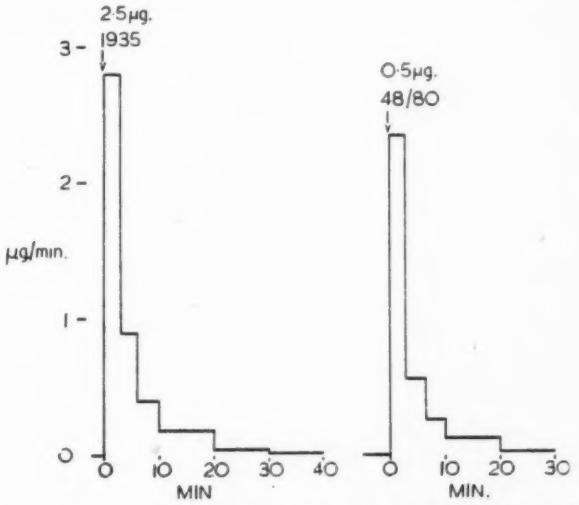



FIG. 1.—Comparison of the rate of output of histamine from perfused skin flaps of cat's hind legs by L 1935 and 48/80. The effect of L 1935 and of 48/80 was obtained from different cats. Ordinates, output of histamine in mg./min. Abscissae: time in min. after injection.

No. 4 shows that 48/80 is more than eight and less than sixteen times as active, and experiment 5 that it is about twelve times as active as L 1935.

The output of histamine is as rapid after L 1935 as after 48/80, and is more gradual with both substances after the second or third injection. In order to compare the output of histamine by 48/80 and L 1935, it is best to use experiments in which the total release of histamine is of the same order. For instance, in experiment No. 1 a first injection of 0.5 μg. 48/80 released 11.6 μg. histamine, and in experiment No. 2 a first injection of 2.5 μg. L 1935 released 15.0 μg. In Fig. 1 the output of histamine in μg./min. for these experiments is plotted against the time of perfusion after the injections in minutes. The samples were collected at successive intervals of 3, 3, 4, 10, and 10 minutes.

As with 48/80, the output of histamine after successive injections of the same dose of L 1935 decreased so much that it was not deemed advisable to give more than three injections to each preparation.

As with 48/80, injections of L 1935 in doses which caused a release of several μg. of histamine were followed by the quick development of oedema which was particularly striking in the loose subcutaneous tissue.

*Perfused Hind Quarters of the Rat*

L 1935 causes a release of histamine from the perfused hind quarters of the rat, but the doses required are higher than for the cat's skin flaps. Table III shows the increasing output of histamine in six perfusion experiments after increasing doses

TABLE III

HISTAMINE RELEASE BY L 1935 FROM PERFU SED HIND QUARTERS OF THE RAT

| L 1935 Injected (μg.) | Output of Histamine (μg.) |
|---|---|
| 10 | 1·38 |
| 50 | 3·19 |
| 50 | 3·69 |
| 50 | 4·15 |
| 100 | 7·18 |
| 200 | 9·36 |

of L 1935. When compared with 48/80 it was found, as in the corresponding experiments on the cat's skin flap, that 48/80 was more active in releasing histamine than L 1935 (Table IV). From experiments 1 and 2 in Table IV it is seen that 50 μg. L 1935 is weaker than 10 μg. 48/80 but stronger than 2.5 μg. Thus, 48/80 is more than five times and less than twenty times as active as L 1935. In the succeeding four experiments the

TABLE IV

COMPARISON OF THE HISTAMINE OUTPUT BY L 1935 AND 48/80 FROM PERFUSED HIND QUARTERS OF THE RAT

| Experiment No. | Injections of 48/80 or L 1935 (μg.) | | Output of Histamine (μg.) |
|---|---|---|---|
| 1 | 50 | L 1935 | 3·19 |
| | 10 | 48/80 | 9·81 |
| | 50 | L 1935 | 0·97 |
| 2 | 50 | L 1935 | 3·69 |
| | 2·5 | 48/80 | 1·73 |
| | 50 | L 1935 | 1·47 |
| | 10 | 48/80 | 1·87 |
| 3 | 10 | L 1935 | 1·38 |
| | 1 | 48/80 | 0·92 |
| | 10 | L 1935 | 0·53 |
| 4 | 50 | L 1935 | 4·15 |
| | 5 | 48/80 | 4·11 |
| | 50 | L 1935 | 1·05 |
| 5 | 5 | 48/80 | 9·17 |
| | 50 | L 1935 | 2·6 |
| | 5 | 48/80 | 1·49 |
| 6 | 100 | L 1935 | 7·18 |
| | 10 | 48/80 | 6·94 |
| | 100 | L 1935 | 4·00 |
| 7 | 200 | L 1935 | 9·36 |
| | 20 | 48/80 | 3·10 |
| | 200 | L 1935 | 0·28 |

ratio between L 1935 and 48/80 was kept at 10:1, but the substances were tested in increasing amounts. Taking into account the fact that the preparations became less sensitive with successive injections, it is evident that, weight for weight, 48/80 is about 10 times as active as L 1935, or perhaps even a little more.

*Effect on Cat's Blood Pressure*

Like compound 48/80, L 1935 caused a fall in blood pressure with the characteristic latency of 20–40 sec. In the experiment of Fig. 2, the latency after the injection of 100 μg. L 1935 was about 40 sec. When the effects of the two substances were compared, it was found that L 1935, in a dose 10 times greater than 48/80, produced a somewhat smaller depressor effect, as illustrated in Fig. 2. A more exact comparison was difficult to obtain in the few experiments carried out

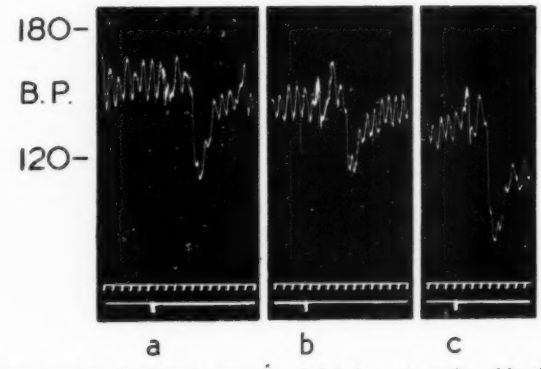



FIG. 2.—Arterial blood pressure of 3.8 kg. cat under chloralose anaesthesia. At *a* and *c* 10 μg. 48/80, at *b* 100 μg. L 1935 intravenously. Time in 10 sec.

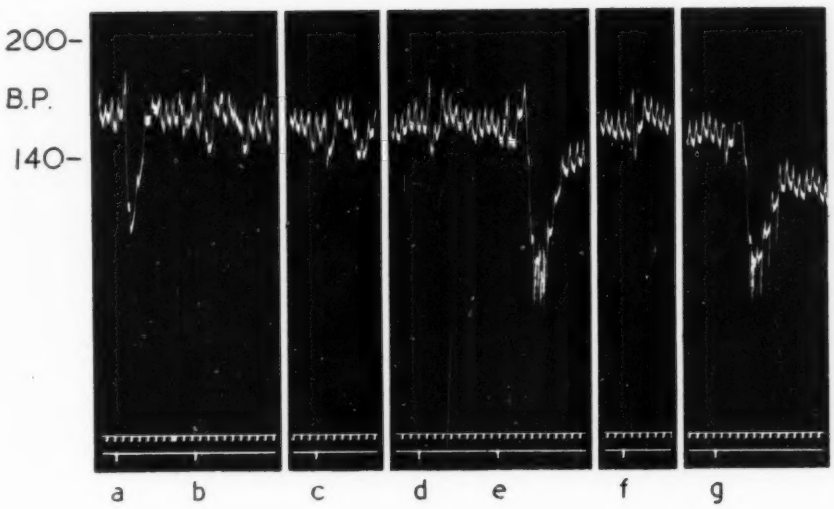



FIG. 3.—Arterial blood pressure of a 3.4 kg. cat under chloralose anaesthesia. Intravenous injections of 0.2 μg. histamine (at *a*), of 15 μg. 48/80 at *b* and *e*, of 150 μg. L 1935 at *c* and *f*, and of 250 μg. L 1935 at *g*; at *d* 2 ml. saline which produced an immediate fall of pressure. Time in 10 sec.

because a second and third injection of the same dose of 48/80 sometimes caused increasing effects, whereas with further injections the blood pressure of the cat became less sensitive to the depressor action of both substances. In the experiment of Fig. 3 the first injection of 15 μg. of 48/80 and the subsequent injection of 150 μg. of L 1935 were subthreshold, but when 15 μg. of 48/80 was injected a second time after the 150 μg. of L 1935 there resulted a sharp fall in arterial blood pressure. A second injection of 150 μg. of L 1935, however, remained subthreshold, whereas 250 μg. caused a fall in blood pressure. This kind of result was obtained in other experiments as well, and it appeared that on repeated alternate injections of the two substances, 48/80 was first about 10 to 12 times, but later about 15 times, as active as L 1925. There was some indication that a first or second injection of a small dose of L 1935, which produced a certain degree of tachyphylaxis to L 1935 itself, increased the sensitivity of the cat to 48/80.

*Effect on the Guinea-pig's Ileum Preparation*

Compound 48/80 is known to have several effects when tested in relatively high concentrations on the isolated guinea-pig's ileum preparation. It produces a strong transient contraction followed, after the 48/80 has been washed out, by a period of increased motor activity and decreased sensitivity to histamine and acetylcholine. The decreased sensitivity may occur whilst the motor activity develops (Mongar and Schild, 1952 ; Dews, Wnuck, Fanelli, Light, Tornaben, Norton, Ellis and de Beer, 1953 ; Feldberg and Smith, 1954).

When 1 mg. of L 1935 was added to the bath the gut showed, after 90 sec., a strong contraction in two out of six preparations. With successive administration of the same dose of L 1935 the contractions became smaller and disappeared. Fig. 4 shows the contraction produced by the first administration of 1 mg. L 1935 to one of these preparations and illustrates the development of strong motor activity after washing out the L 1935. Motor activity developed also when there was no direct stimulating effect of L 1935 on the ileum preparation. In Fig. 5, which illustrates the depressing action of L 1935, the development of increased motor activity after the washing out is visible, although there was no direct stimulation.

After L 1935 is washed out the preparation becomes less sensitive not only to histamine but also to acetylcholine. When the depression is pronounced, full recovery seldom occurs.

When the depressing effect of L 1935 was compared with that of 48/80 the two compounds were

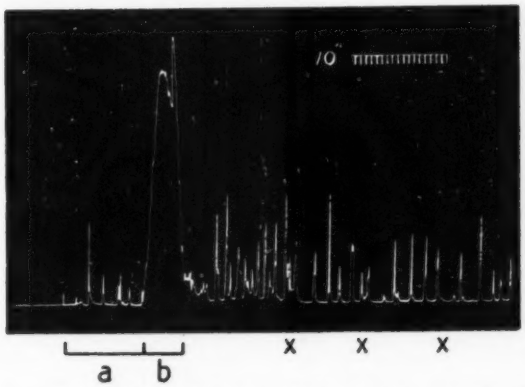



FIG. 4.—Guinea-pig's ileum preparation in 15 ml. magnesium-free Tyrode solution; 0.02 μg. atropine sulphate throughout. At *a* 500 μg. and at *b* 1 mg. of L1935 kept in the bath for 90 sec. Time in 10 sec. At each x the Tyrode solution of the bath was renewed.

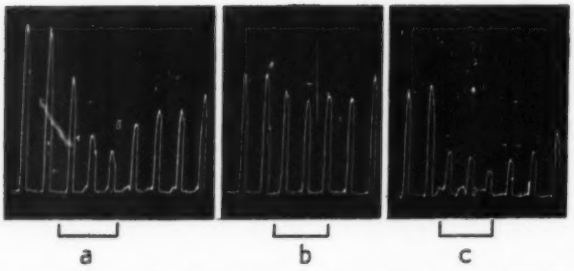



FIG. 5.—Guinea-pig's ileum preparation in 15 ml. magnesium-free Tyrode solution; 0.02 μg. atropine throughout. Contractions due to 0.06 μg. histamine added to the bath for 15 sec. at 1 min. intervals. At *a* 1 mg. of L 1935, at *b* 200 μg. and at *c* 1 mg. of 48/80 were added to the bath. The additions were made 30 sec. before three successive histamine injections, as indicated at the bottom of the figure.

found to be about equally active, weight for weight. This is illustrated in Fig. 5, which shows that 200 μg. of 48/80 produces a much weaker depression than 1 mg. of L 1935, but that the depressions produced by 1 mg. of 48/80 and by 1 mg. of L 1935 are comparable.

As these actions of L 1935 were obtained with much larger doses than those present in the amounts of venous effluent used when testing for histamine, they did not interfere with the assay.

## DISCUSSION

L 1935 was found to be a potent histamine liberator when injected into perfused flaps of the cat's skin or into the perfused hind quarters of the rat. The time course of the release of histamine was found to be about the same for both substances; therefore the release after L 1935 is probably as "explosive" as that after 48/80.

On the perfused skin preparations, doses of L 1935 as small as 1 or even 0.5 μg. caused the release of detectable amounts of histamine. Although L 1935 is not as potent as compound 48/80, it is more potent on these perfused tissues than any other of the known histamine liberators. For instance, the potent histamine releasers propamidine and octylamine are 100–200 times less active on the perfused cat's skin flap than 48/80, whereas L 1935 was found to be only about 10 times less active. Feldberg and Mongar (1954) have emphasized that when a comparison is made between two histamine liberators different ratios can be obtained according to the species, tissues, and methods used. This fact has to be kept in mind when stating that compound 48/80 is about 10 times as active as L 1935 in releasing histamine.

The depressor action we observed on the cat's arterial blood pressure after the intravenous injections of relatively small doses of L 1935 seems to be fully accounted for by released histamine. The fact that the blood pressure did not fall immediately after the injection but only after a latency of 20–40 sec. speaks against a direct depressor action of L 1935 in these experiments. Further, L 1935 was 10 to 15 times less active in causing a fall of blood pressure than 48/80; this has to be compared with the ratio of about 1:10 for the histamine-releasing activity of the two compounds in the perfusion experiments and strongly suggests that release of histamine is the sole cause of the depressor action we observed. The conclusion need not necessarily apply to the depressor effect of larger doses of either 48/80 or L 1935.

The progressive diminution in the depressor action of L 1935 when injected repeatedly into the cat cannot be explained by a gradual depletion of the histamine stores of the tissues, because it occurred after relatively small doses. These can have caused the release of only a very small fraction of the available histamine. The decreased sensitivity is thus a tachyphylaxis which concerns the mechanism of release. There was some indication that this tachyphylaxis developed more rapidly after the intravenous injections of L 1935 than after those of 48/80, but more experiments would be needed to make certain about this difference.

One of the problems presented by histamine liberators in general is how far release of histamine accounts for all the observed effects. It seems safe to conclude that this is so with regard to the itching, the erythema, the urticaria, the facial oedema, and the fall in arterial blood pressure observed in man after intravenous injections of L 1935. However, large doses of L 1935 injected into animals may well give effects which are not accounted for by histamine release. On the guinea-pig's intestine such effects certainly occur after L 1935, and it is interesting to note that strikingly similar effects are obtained with other histamine liberators as well. The potency of L 1935 in depressing the sensitivity of the guinea-pig's ileum was found to be about as great as that of 48/80, in contrast to the ratio of 1:10 obtained for the histamine-releasing property of the two compounds when tested on perfused tissues of the cat and the rat. Their histamine releasing property has not been compared on the guinea-pig's ileum, and on this tissue the ratio may be different. There is, however, no necessity to assume that the depression observed with both substances is related to their potency in releasing histamine. Histamine-releasing substances seem to have other properties in common which are not related to their potency in releasing histamine. For instance,

Gertner (1955) recently observed that 48/80 as well as propamidine caused block of ganglionic transmission; but the doses required to produce this effect appeared to bear no relation to the potency of the two compounds in releasing histamine.


## Summary

1. L 1935 causes a release of histamine from perfused skin flaps of the cat's hind legs and from perfused hind quarters of the rat. Weight for weight it is 10–12 times less active than compound 48/80.

2. L 1935, when injected intravenously into cats, causes a fall in arterial blood pressure after a latency of about 20–40 sec. Weight for weight it is 10 to 15 times less active than 48/80. On repeated injections the cat becomes progressively less sensitive to the depressor action of L 1935.

3. L 1935 produces on the isolated guinea-pig's ileum, when added to the bath in relatively high concentration, transient contraction followed, after washing out, by a period of increased motor activity and decreased sensitivity to histamine and acetylcholine. Similar effects are produced by 48/80. Weight for weight the two substances are about equally active on the guinea-pig's ileum.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# STUDIES ON THE MECHANISM OF PITUITARY-ADRENAL ACTIVATION BY MORPHINE

BY

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That a relationship exists between the effect of morphine and the activity of the adrenal cortex was first shown by Lewis (1920), who observed that adrenalectomized rats were four to five hundred times more sensitive to morphine than were the control rats. MacKay and MacKay (1926) found that the adrenal cortex hypertrophied when a rat was treated with increasing doses of morphine. Similar results have been observed by Sung, Way, and Scott (1953) while studying rats made tolerant to morphine and methadone ; however, the integrity of the adrenals does not appear to be essential in the development of chronic tolerance to the narcotic analgesics (Way, Sung, and Fujimoto, 1954). Recently, Nasmyth (1954) reported that adrenal ascorbic acid depletion occurred in rats following a single dose of morphine.

Since numerous other agents have been shown to deplete adrenal ascorbic acid, and since there appear to be gaps in the knowledge as to the manner in which such compounds exert this effect, studies were initiated in normal, adrenal demedullated and hypophysectomized rats to investigate the relative degree of specificity of the adrenal response to morphine as well as to methadone, and to assess the possible mechanisms by which these drugs stimulate the pituitary-adrenal axis.

## METHODS

*Administration of Drugs.*—Male rats of the Sprague-Dawley strain, weighing 130–200 g., were used. They were maintained on a diet of Purina Lab Chow and tap water. The doses of morphine and of the *dextro* and *laevo* isomers of methadone were selected as fractions of the LD50. The LD50 of each drug was determined by the method of Litchfield and Wilcoxon (1949). The compounds were dissolved in distilled water and injected subcutaneously in the test animals, and the controls were injected with equal volumes of distilled water.

*Estimation of Adrenal Ascorbic Acid.*—All animals were killed by decapitation 90 min. after drug administration. Both adrenals of each animal were removed, dissected free of fat and weighed to 0.5 mg. The glands were then homogenized in 10 ml. of 4% trichloroacetic acid, and the ascorbic acid was determined by Geschwind, Williams, and Li's (1951) modification of the method of Roe and Kuether (1943).

*Demedullation of Adrenal Glands.*—The procedure was carried out according to the technique described by Evans (1936). All animals were given 0.5% saline for seven days following surgery, and were then given tap water. The groups studied in the morphine experiments were injected and sacrificed 21 days following demedullation ; in the methadone studies the post-operative interval was 32–40 days. One group of animals was selected for histological examination. The adrenals of this group were fixed in formalin, sectioned serially at 7 $\mu$, stained with haematoxylin and eosin, and examined microscopically for adrenal medullary tissue.

*Hypophysectomy.*—Animals were hypophysectomized by the parapharyngeal approach and were examined five days later.

## RESULTS

*Toxicity and Effect on Adrenal Ascorbic Acid in Normal Rats.*—The acute toxicity and effect of morphine, (−)- and (+)-methadone on adrenal ascorbic acid content are summarized in Table I. All three compounds produced a significant depletion at 1/5 LD50 but not at 1/15 LD50 ; (−)-methadone gave the most pronounced effect and (+)-methadone the least. The results with (+)-methadone were not nearly as uniform as with morphine or (−)-methadone, and attempts to increase the response to (+)-methadone by increasing the dosage to as high as 1/2 LD50 resulted in little or no enhancement of its adrenal ascorbic acid depleting effect.

It is of interest that our toxicity data indicate that *laevo*methadone given subcutaneously is approximately 5.5 more toxic than the *dextro*-isomer, particularly since other investigators (Scott, Robbins, and Chen, 1948 ; Thorp, 1949) have

TABLE I

TOXICITY AND EFFECT OF MORPHINE, (−)- AND (+)-METHADONE ON THE ADRENAL ASCORBIC ACID CONTENT OF NORMAL RATS FOLLOWING SUBCUTANEOUS ADMINISTRATION

| Compound | LD50 and 19/20 Confidence Limits | No. of Rats | Dose | | Adrenal Ascorbic Acid | | P |
|---|---|---|---|---|---|---|---|
| | | | Mg./kg. | Approx. Fraction of LD50 | Mean mg. % ±S.E. | % of Control | |
| Distilled water .. .. | — | 18 | — | — | 489±17 | — | — |
| Morphine sulphate .. .. | 108 (80·2–146·0) mg./kg. | 6 | 22·0 | 1/5 | 324±34 | 66·4 | 0·001 |
| | | 6 | 8·0 | 1/15 | 433±19 | 88·7 | 0·1 |
| (−)-Methadone hydrochloride | 20 (5·3–26·2) mg./kg. | 6 | 4·0 | 1/5 | 280±15 | 57·4 | 0·001 |
| | | 6 | 1·4 | 1/15 | 433±14 | 88·7 | 0·1 |
| (+)- „ „ | 112 (75·0–166·9) mg./kg. | 3 | 60·0 | 1/2 | 357±60 | 73·0 | 0·01 |
| | | 10 | 30·0 | 1/4 | 380±21 | 77·7 | 0·01 |
| | | 6 | 22·0 | 1/5 | 387±29 | 79·3 | 0·01 |
| | | 6 | 8·0 | 1/15 | 453±18 | 92·8 | 0·3 |

shown that the two isomers have approximately the same LD50 in mice when injected intravenously. In the latter instance the identical LD50s appear to be fortuitous since apparently the isomers kill by different mechanisms. Our unpublished observations indicate that nalorphine antagonizes the toxic effects of *laevo*methadone but not of *dextro*methadone.

TABLE II

EFFECT OF MORPHINE, (−)- AND (+)-METHADONE ON THE ADRENAL ASCORBIC ACID CONTENT OF HYPOPHYSECTOMIZED RATS FOLLOWING SUBCUTANEOUS ADMINISTRATION

| Compound | No. of Rats | Dose (mg./kg.) | Adrenal Ascorbic Acid | | P |
|---|---|---|---|---|---|
| | | | Mean mg. %±S.E. | % of Control | |
| Distilled water .. | 8 | — | 433±33 | — | — |
| Morphine sulphate | 5 | 22·0 | 454±50 | 103·0 | 0·7 |
| (−)-Methadone hydrochloride .. | 5 | 4·0 | 422±26 | 96·0 | 0·8 |
| (+)-Methadone hydrochloride .. | 4 | 22·0 | 410±19 | 93·2 | 0·6 |

*Effect on Adrenal Ascorbic Acid in Hypophysectomized Rats.*—It can be seen from Table II that doses of morphine, and of the methadone isomers, which deplete adrenal ascorbate in normal rats are ineffective in hypophysectomized animals, thus indicating that integrity of the adenohypophysis is essential for this effect.

*Effect on Adrenal Ascorbic Acid in Demedullated Rats.*—Bilateral adrenal medullectomy did not impair the adrenal cortical response to morphine or the methadones. In fact, the surgical procedure may have enhanced the effect, since the compounds depleted adrenal ascorbate at doses much lower than those required in normal animals (Table III). With morphine a significant depletion was obtained with 1/10 of the dose that was effective in normal rats. However, Nasmyth (1954) reported that the same dose of morphine

TABLE III

EFFECT OF MORPHINE, (−)- AND (+)-METHADONE ON THE ADRENAL ASCORBIC ACID CONTENT OF DEMEDULLATED RATS FOLLOWING SUBCUTANEOUS ADMINISTRATION

| Compound | No. of Rats | Mean Adrenal Weight (mg.) | Dose (mg./kg.) | Adrenal Ascorbic Acid | | P |
|---|---|---|---|---|---|---|
| | | | | Mean mg. %±S.E. | % of Control | |
| Controls for morphine ($H_2O$) | 6 | 19·4 | — | 307±29 | — | — |
| Morphine sulphate | 6 | 19·5 | 8·0 | 207±9 | 67·5 | 0·01 |
| | 6 | 18·8 | 4·0 | 234±18 | 76·2 | 0·05 |
| | 6 | 19·3 | 2·0 | 234±10 | 76·2 | 0·05 |
| Controls for methadone ($H_2O$) | 5 | 26·6 | — | 547±24 | — | — |
| (−)-Methadone hydrochloride | 6 | 27·8 | 1·4 | 395±46 | 72·2 | 0·02 |
| | 6 | 26·5 | 0·7 | 526±26 | 96·0 | 0·3 |
| (+)-Methadone hydrochloride | 6 | 24·9 | 8·0 | 466±19 | 85·3 | 0·05 |
| | 6 | 27·6 | 4·0 | 574±26 | 104·5 | 0·5 |

reduced adrenal ascorbic acid in normal and demedullated rats to approximately the same degree. It cannot be stated for certain whether our findings indicate a true enhancement of the adrenal ascorbate response since the demedullated animals appeared to be more sensitive to the toxic effects of morphine and LD50 determinations were not made in this group. However, the results indicate clearly that adrenal medullary discharge of adrenaline does not play a major role in effecting adrenal ascorbic acid depletion with the above compounds.

It would appear from these data that morphine is relatively more specific than the methadones, but it is not possible to compare the effect of morphine and the methadones on the adrenal ascorbate response in medullectomized animals because experimental conditions were not identical. The groups of animals in the morphine study were used only 21 days post-operatively, and therefore were probably more sensitive than those used in the methadone studies in which the post-operative interval was 32 to 40 days. The difference in recovery period gives a plausible explanation for

the disparity in the ascorbic acid content and adrenal weight of the control values in the two studies. This conclusion is supported by the report of Greep and Deane (1949) that the adrenal ascorbic acid concentration becomes constant and is present in all of the cortical cells in normal amounts about the 32nd day after enucleation.

*Effect of Morphine, Aspirin, and Adrenaline on the Adrenal Ascorbic Acid of Rats Pretreated with Cortisone.*—In order to elucidate the mechanism by which morphine depletes adrenal ascorbic acid, experiments were undertaken to determine whether this effect was due to an increased cellular utilization of circulating adrenal corticoids or to some other mechanism. The circulating level of cortisone was elevated by injecting animals with 5 or 10 mg./kg. of cortisone three hours before the administration of morphine. It can be seen from Table IV that neither of these doses of cortisone abolished the fall in adrenal ascorbic acid following morphine, although the response was partially

TABLE IV

EFFECT OF MORPHINE, ASPIRIN, AND ADRENALINE ON THE ADRENAL ASCORBIC ACID CONTENT OF RATS PRETREATED WITH CORTISONE ACETATE

| Compound | No. of Rats | Dose (mg./kg.) | Adrenal Ascorbic Acid | | P |
|---|---|---|---|---|---|
| | | | Mean mg. %±S.E. | % of Control | |
| a. Cortisone (5 or 10 mg./kg.) distilled water | 8 | | 449±21 | 100 ±4·7 | — |
| b. Morphine sulphate .. | 8 | 30 | 266±8 | 59·4±1·8 | 0·001 (a–b) |
| c. Cortisone (5 mg./kg.)+ morphine | 10 | 30 | 362±20 | 80·6±4·6 | 0·01 (a–c)<br>0·001 (b–c) |
| d. Cortisone (10 mg./kg.)+ morphine | 10 | 30 | 371±17 | 82·7±3·8 | 0·01 (a–d)<br>0·001 (b–d) |
| e. Aspirin .. | 8 | 150 | 280±16 | 62·4±3·6 | 0·001 (a–e) |
| f. Cortisone (5 mg./kg.)+ aspirin | 6 | 150 | 285±16 | 63·5±3·6 | 0·001 (a–f)<br>0·9 (e–f) |
| g. Cortisone (10 mg./kg.)+ aspirin | 6 | 150 | 354±40 | 78·8±8·9 | 0·05 (a–g)<br>0·07 (e–g) |
| h. Adrenaline hydrochloride | 4 | 0·1 | 322±5 | 71·7±1·1 | 0·001 (a–h) |
| i. Cortisone (5 mg./kg.)+ adrenaline | 10 | 0·1 | 322±5 | 71·7±1·1 | 0·001 (a–i)<br>1·0 (h–i) |
| j. Cortisone (10 mg./kg.)+ adrenaline | 8 | 0·1 | 363±25 | 80·8±5·6 | 0·02 (a–j)<br>0·2 (h–j) |

blocked. Neither did cortisone pretreatment block the response to aspirin or adrenaline, though the 10 mg. dose of cortisone appeared to have some inhibitory effect. These results are not in harmony with those of Sayers and Sayers (1947), who reported that 1.0 mg./kg. of cortisone blocked the ascorbic acid depleting effect of adrenaline in unilaterally adrenalectomized animals under anaesthesia. Under our experimental conditions we were unable to demonstrate any blocking effect to morphine, aspirin, or adrenaline with a 1.0 mg./kg. dose of cortisone. Our findings are supported by those of Cronheim and Hyder (1954), who found that doses of cortisone as large as 80 mg./kg. failed to abolish the adrenal cortical response to aspirin.

*Effect of Morphine and Aspirin on Adrenal Ascorbic Acid in Rats Pretreated with Nalorphine.*—All animals were treated with 5 mg./kg. nalorphine subcutaneously 5 min. before the injection of morphine, (−)-methadone, (+)-methadone, or aspirin. It can be seen from Table V that nalorphine in this dose, which does not significantly

TABLE V

EFFECT OF MORPHINE, (−)- AND (+)-METHADONE AND ASPIRIN ON THE ADRENAL ASCORBIC ACID CONTENT OF RATS PRETREATED WITH NALORPHINE

| Compound | No. of Rats | Dose (mg./kg.) | Adrenal Ascorbic Acid | | P |
|---|---|---|---|---|---|
| | | | Mean mg. %±S.E. | % of Control | |
| a. Distilled water | 10 | — | 450±21 | 100 ±4·7 | — |
| b. Nalorphine hydrochloride | 18 | 5 | 415±11 | 92·4±2·4 | 0·1 (a–b) |
| c. Morphine sulphate .. | 8 | 30 | 266±8 | 59·2±1·8 | 0·001 (a–c) |
| d. Nalorphine+ morphine | 9 | 5<br>30 | 360±13 | 80·0±2·9 | 0·001 (a–d)<br>0·01 (b–d)<br>0·001 (c–d) |
| e. (−)-Methadone hydrochloride | 8 | 6 | 326±14 | 72·4±3·1 | 0·001 (a–e) |
| f. Nalorphine+ (−)-methadone | 9 | 5<br>6 | 398±18 | 88·5±4·0 | 0·1 (a–f)<br>0·3 (b–f)<br>0·01 (e–f |
| g. (+)-Methadone hydrochloride | 10 | 30 | 380±21 | 84·3±4·7 | 0·05 (a–g) |
| h. Nalorphine+ (+)-methadone | 11 | 5<br>30 | 368±12 | 81·8±2·7 | 0·01 (a–h)<br>0·01 (b–h)<br>0·5 (g–h) |
| i. Aspirin .. | 8 | 150 | 280±16 | 62·2±3·6 | 0·001 (a–i) |
| j. Nalorphine+ aspirin | 8 | 5<br>150 | 274±19 | 61·0±4·2 | 0·001 (a–j)<br>0·001 (b–j)<br>0·8 (i–j) |

lower the adrenal ascorbic acid content, produced a pronounced inhibition of the ascorbic acid depleting effect of morphine and (−)-methadone but not of (+)-methadone and aspirin.

## DISCUSSION

The data clearly indicate that morphine and *laevo*methadone deplete adrenal ascorbic acid and that the integrity of the pituitary is essential for this effect. The precise mechanism by which these compounds mediate an action on the pituitary is in need of clarification. Conceivably, morphine activates the anterior pituitary gland to release ACTH by one or more of several mechanisms which may be independent or interdependent: (*a*) a reflex secretion of endogenous adrenaline

which then acts on the pituitary; (*b*) a release of tissue histamine; (*c*) a fall in the level of circulating adrenal cortical hormones as a result of increased cellular utilization; (*d*) a direct action on the anterior pituitary; and (*e*) a neural or neuro-humoral discharge in the hypothalamus.

Although our results do not completely exclude the first three possibilities, it is unlikely that morphine acts by one of these mechanisms. The results with demedullated rats show conclusively that the adrenal response to morphine and methadone occurs in the absence of the release of adrenal medullary adrenaline. Moreover, an earlier study indicated that the dose of morphine required to effect adrenal medullary discharge of adrenaline in the rat, as measured by the hyperglycaemic response, is at least double that required in the present study to deplete adrenal ascorbic acid (Miller *et al.*, 1955). These data are supported by the results of Nasmyth (1954), who reported a depletion of adrenal ascorbic acid in demedullated rats following morphine administration. In the same study Nasmyth showed that tissue release of histamine following morphine was not an important factor in depleting adrenal ascorbic acid. Finally, an increase in the level of circulating cortisone failed to block the release of ACTH following the injection of morphine.

In light of recent evidence that the adrenocortical response to stress may be controlled by the hypothalamus (DeGroot and Harris, 1950; Hume and Wittenstein, 1950; Porter, 1952; McCann, 1953; Ganong and Hume, 1954), it seems more likely that morphine acts via a neural or neurohumoral mechanism. Experiments are at present in progress to investigate this effect of morphine in rats with hypothalamic lesions. Evidence that morphine acts on the hypothalamus has been demonstrated by McCrum and Ingram (1951), who found that cats with lesions in the caudal hypothalamus failed to exhibit the characteristic symptoms of hyperthermia and hyperactivity following morphine administration. Further evidence is indicated by the antidiuretic effect of morphine in dogs (DeBodo, 1944) and rats (Giarman, Mattie, and Stephenson, 1953; Winter, Gaffney, and Flataker, 1954). DeBodo reported that morphine, while producing a marked antidiuretic effect in normal dogs, failed to inhibit water diuresis caused by pituitary stalk section or hypophysectomy; Winter *et al.* have shown that the antidiuretic effect of morphine is effectively blocked by nalorphine and the urine of morphine-treated rats contains an antidiuretic substance which is not morphine, and which is not antagonized by nalorphine. More recently, the results of McCann and Brobeck (1954) indicate that the supraopticohypophyseal tract may play a role in the regulation of ACTH secretion by release of antidiuretic hormone into the hypophyseal portal vessels. They reported that destruction of the supraopticohypophyseal tract blocks pituitary ACTH release in response to stress and that ascorbic acid depletion, which normally follows stress, failed to occur in rats with severe diabetes insipidus. These data make it attractive to speculate that the site of action of morphine, which evokes ascorbic acid depletion and antidiuresis, may be one and the same.

Inasmuch as a variety of stresses and a variety of compounds can evoke adrenal ascorbic acid depletion, the question arises, Can this action of morphine and methadone be merely a non-specific effect? It would seem apparent from our results that the adrenal ascorbic acid depleting effect of morphine—or of any other compound acting systemically—cannot arbitrarily be classified as non-specific. Not only is the specificity of the response relative in nature but the mechanisms concerned with mediating the effect may be different. Our studies clearly show that nalorphine, a specific morphine antagonist, blocks the adrenal ascorbic acid response in rats injected with morphine but fails to inhibit this effect following aspirin administration. This strongly suggests that different receptors or sites are concerned with mediating this effect for morphine and aspirin as well as for (−)-methadone and (+)-methadone. Evidence that morphine may act differently from other chemical agents is also indicated by the studies of Briggs and Munson (1954), who reported that the adrenal ascorbic acid depleting effect of morphine but not of histamine, pitressin, and adrenaline can be suppressed by previous injection of pentobarbitone sodium.

It is clear, therefore, that pharmacological agents can effect depletion of adrenal ascorbic acid by one or more of several mechanisms. Rather than characterize an agent by a mechanism which remains to be elucidated, it appears more appropriate to define specificity of action of the agent in relative terms from quantitative data obtained by standardized pharmacological techniques. Thus, until the information becomes available, the relative specificity of the response by which an agent causes depletion of adrenal ascorbic acid can better be described in terms of (*a*) its minimal effective dose, (*b*) the potency ratio of the compound in relation to its toxicity, and (*c*) whether or not other pharmacological effects are evident with a dose of the compound which evokes adrenal ascorbic acid depletion.

SUMMARY

1. The acute toxicity and effect of morphine, and of (−)- and (+)-methadone on adrenal ascorbic acid were studied in normal rats. All three compounds produced a significant fall in the ascorbic acid content of rat adrenals at 1/5 LD50 but not at 1/15 LD50.

2. Doses of morphine and (+)- and (−)-methadone, which deplete adrenal ascorbic acid in normal rats, were ineffective in hypophysectomized animals.

3. Bilateral adrenal demedullation did not impair the adrenal cortical response to morphine and (+)- or (−)-methadone. In fact, the compounds depleted adrenal ascorbic acid at doses much lower than those required in normal animals.

4. Pretreatment with cortisone (5 or 10 mg./kg.) did not prevent a reduction in adrenal ascorbic acid following morphine, aspirin, or adrenaline, but did modify the response.

5. Pretreatment with nalorphine produced a pronounced inhibition of the ascorbic acid depleting effect of morphine and (−)-methadone but not of (+)-methadone and aspirin. This suggests that different receptors or sites are concerned with mediating this effect, possibly in the hypothalamus.

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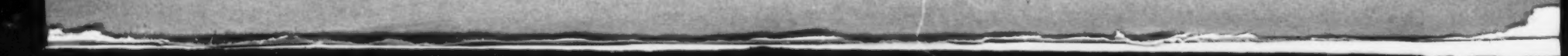

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